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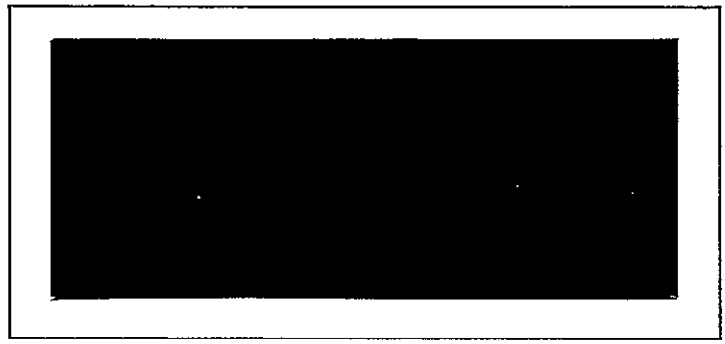

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Report

"TECHNOLOGY FOR RETURN OF PLANETARY SAMPLES"

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1.0 SUMMARY

This report presents recent progress on the development of a Basic Warning System (BWS) proposed to assess the biohazard of a Mars sample returned to Earth, an Earth orbiting spacecraft or to a moon base. The BWS package consists of terrestrial microorganisms representing major metabolic pathways. A "Vital Processes" component of the BWS will examine the effects of a Mars sample at terrestrial atmospheric conditions while a "Hardy Organism" component will examine the effects of a Mars sample under conditions approaching those of the Martian environment. Any deleterious insult on terrestrial metabolism effected by the Mars sample could be indicated long before the sample reached Earth proximity.

During the past year, the BWS has been developed as a laboratory model for a flight experiment and conditions defined for monitoring indicator organisms. For the Vital Processes system, emphasis was placed on refinement of techniques to monitor heterotrophic metabolism and a technology was developed whereby both increases and decreases in the level of ^{14}C-labeled gas in the headspace above a reaction mixture can be monitored. Using this system, conditions have been refined to optimize metabolic responses resulting from repeated additions of substrate at various time intervals.

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Although the major program emphasis was development of techniques for monitoring heterotrophic metabolism, methods required to monitor other metabolic pathways have also been defined. The heterotrophic technique has been successfully utilized to monitor metabolism of nitrogen-fixing microorganisms. Growth and metabolism of such organisms occurs in the absence of nitrate or other added nitrogen source through fixation of atmospheric nitrogen. Without such fixation, organisms cannot use carbon to metabolize and grow in a nitrogen-free medium. Thus, the test can be used as an indirect monitor of nitrogen fixation.

Standard procedures have also been developed to monitor metabolism of photosynthetic organisms and the method combined with those for monitoring heterotrophic organisms to demonstrate the feasibility of using both types of organisms in an elementary ecosystem. For the Hardy Organism system, conditions approaching those on Mars still allow marginal metabolism of the indicator organism. Although these conditions are not yet optimized in their approximation of the Martian environment, they nonetheless have provided data to support the concept that further experimentation can develop such a system.

To demonstrate the capability of the BWS to detect effects of a Mars sample, a series of experiments has been

conducted whereby inhibitors of various types have been introduced to challenge three of the indicator organisms. Stress agents examined were organisms which compete for a common substrate, predators which could consume the indicator organisms, specific metabolic inhibitors, and stresses resulting from physical properties of the Martian soil. The results, in general, demonstrate the sensitivity of the proposed BWS in detecting the various stresses. However, in some cases no inhibition of metabolism by indicators was detected in the presence of challenging living organisms or known metabolic inhibitors. Thus, the results emphasize the need to consider negative BWS results with caution. They also support the concept of utilizing multiple indicator organisms to increase the probability of observing extraterrestrial effects.

A prerequisite for adapting the proposed BWS to space travel is a means for culture preservation. Methods for long-term preservation by maintaining a viable culture are generally not desirable because of the requirements for continual culture transfers and general maintenance, and the possibility of culture alteration through mutation. However, one approach to active culture maintenance that may be feasible is through the use of the repeated substrate technique developed to monitor heterotrophic metabolism.

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In one experiment, a culture was successfully maintained for four months by adding 0.05 ml aliquots of ^{14}C-labeled lactate at one week intervals. The technique eliminates the need for culture transfer and large quantities of culture medium, and, also, has the advantage of establishing a "response library" over a long duration of time. However, it cannot eliminate the potential problem of culture mutation.

An alternate method for culture preservation that eliminates many difficulties is preservation in a state of "suspended animation". Of the methods available, lyophilization appears most desirable. Experiments have been conducted to optimize culture recovery while minimizing the nutrient requirements in which the culture must be both lyophilized and resuspended. The methods have been successfully applied to two heterotrophs, one nitrogen-fixing organism, and to the Hardy Organism system. However, alternate methods for preserving phototrophs must be sought. Some preliminary experiments indicate that preservation of phototrophs may be possible in an ecosystem environment.

In the continued development of the proposed BWS, it is recommended that the laboratory effort continue to refine monitoring methods with emphasis on those for the Hardy Organism system. The capabilities for delineating effects of stress challenges should be more fully explored and a

means established for long term preservation of phototrophs. It is also recommended that the available Test Standards Module for the Labeled Release experiment be modified to serve as a BWS breadboard and plans are presented to accommodate this modification.

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2.0 INTRODUCTION

The Basic Warning System (BWS) presents a method for determining the possible impact of extraterrestrial life forms on terrestrial microorganisms through investigating the effects on key terrestrial biological processes. Any interaction between the two life systems can serve as an alert, and experiments under controlled conditions will permit preliminary assessment of the hazard that might result from back contamination of Earth.

Two different systems, the Vital Processes System and the Hardy Organism System, have been proposed as single or joint candidates for the BWS. The purpose of the Vital Processes System is to expose basic metabolic cellular processes to the Mars organisms under terrestrial conditions to determine the impact of the foreign organisms on the terrestrial biosphere. Major terrestrial processes, namely heterotrophism, photosynthesis, and nitrogen fixation, are included. The purpose of the Hardy Organism System is to provide conditions as similar as possible to those found on Mars, thus promoting the growth of Mars life forms and permitting the expression of any effects. Terrestrial test organisms that can withstand the rigorous conditions would be used in this system.

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During the previous year, in preparation for the effort reported herein, candidate organisms were screened (1) to select those best suited for further development of the BWS. Candidates for the Vital Processes System were selected to represent specific metabolic pathways: Pseudomonas was selected as a heterotroph, Azotobacter as a nitrogen fixer, and Nostoc as a photosynthetic algae. In addition to individual strains, model ecosystems with combinations of organisms were considered as sensitive and reliable indicators of a potential biohazard. Of these candidates, those displaying bacterial-algal interactions were considered most feasible for further development. For the Hardy Organisms System, the non-purple photosynthetic Rhodospirillum rubrum was chosen. This versatile organism is capable of photosynthesis, anaerobic respiration, and fermentative growth and was demonstrated (1) to be quite tolerant of various harsh environmental conditions.

Of the many monitoring methods considered for a Biological Warning System utilizing microorganisms, the use of ^{14}C compounds was selected as most practical. Heterotrophic metabolism could be monitored by the Labeled Release technique whereby ^{14}C-labeled organic substrates are metabolized to $^{14}CO_2$. Photosynthetic metabolism could be monitored by uptake of $^{14}CO_2$. The ^{14}C techniques are extremely sensitive and Biospherics' personnel have extensive in-house experience

in this technology. The fully automated Labeled Release experiment on Mars (2-4) successfully monitored heterotrophic metabolism and large portions of this technology are readily adaptable to the proposed BWS.

In the current year, the organisms and monitoring systems selected for the BWS have undergone further refinement. Early work in developing the Labeled Release experiment (5,6) made extensive use of the "getter" technique whereby evolved gas is cumulatively trapped over timed increments to determine the kinetics of evolution. However, the getter technique is not an equilibrium system nor can it be used to monitor decreases in gas levels. During a mission, it would be far more advantageous to utilize continuous monitoring of the radioactive gas present in the headspace of a reaction vial. This method is versatile in monitoring all ^{14}C-labeled gases and also makes use of available Viking technology. Thus, in our continued development of a BWS, we have devised laboratory systems for continuous gas monitoring. These systems, more representative of monitoring during a mission, have been utilized to further refine and optimize the proposed Vital Processes and Hardy Organism Systems of the proposed BWS and to demonstrate the effectiveness of the BWS in detecting a potential biohazard. The latter effort has resulted in preliminary establishment of library responses characteristic of different types of stress, and the delineation of the effort required for

completion of such a library. As part of the effort to adapt the proposed BWS to an actual mission, efforts have also been directed towards means of transporting indicator cultures and towards conceptual designs for a functional breadboard.

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3.0 REFINEMENT OF BASIC WARNING SYSTEM

The basic warning system to be sent to Mars will monitor stresses to vital terrestrial processes under terrestrial conditions and of terrestrial organisms under conditions more closely approximating those of Mars. Techniques to monitor these processes have been refined and are reported in the sections below.

3.1 Vital Processes Techniques

The vital processes selected for refinement are heterotrophic and photosynthetic metabolism. Techniques for monitoring heterotrophs are also applicable to monitoring nitrogen fixing organisms thereby indirectly monitoring nitrogen fixation. The standard procedures used for each technique along with development of various aspects of the method refinements are presented below.

3.1.1 Heterotrophic Metabolism

3.1.1.1 Standard Procedures: Standard bacterial cultures for use in heterotrophic experiments were Pseudomonas fluorescens and Ps-3, an isolate probably identical with P. fluorescens. These cultures were grown on lactate minimal medium of the following composition:

Medium A: Lactate Minimal Medium	
Sodium Lactate	0.1% (W/V)
$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4

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Cells were grown for 48 hours in this medium, after which a loopful was inoculated into a second tube for an additional 48 hours' growth. All cultures were contained in 10 ml screw cap tubes which were shaken on a Blue M Co. reciprocating shaker (at a setting of 15) at room temperature (app. 26°C). The final inoculum was diluted from the second 48 hour culture with an inorganic medium of the following composition:

Medium B: Pseudomonas and Ps-3 Dilution Medium

$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4

The inorganic composition of this medium is identical to that containing lactate (Medium A). Dilutions were performed by optical density measurements to obtain an inoculation concentration for each vial of 10^3 cells/0.05 ml dilution medium.

Heterotrophic metabolism is monitored by the Labeled Release technique whereby ^{14}C-labeled organic substrates are added to a culture placed on a 0.5 g silica base and the incubation mixture monitored for evolution of ^{14}C-labeled carbon dioxide. The technique selected to perform such experiments is that devised by Buddemeyer (7) using a double vial system to monitor $^{14}CO_2$ evolution.

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The technique uses vials to supply two compartments: The outer compartment is a standard liquid scintillation glass vial (28 mm O.D. x 58 mm h). A smaller inner vial serves as the culture chamber. Depending on the amount of medium added, either a 10 mm O.D. x 35 mm h tube size with a 2 ml capacity or a 15 mm O.D. x 45 mm h tube size with a 4 ml capacity is used. A fluor-coated getter is inserted between the inner and outer vials. Radioactive gas produced in the culture chamber migrates to the outer vial where beta particles from ^{14}C disintegrations may strike the fluor-coated paper. Light produced by such events is detected by the phototubes of the liquid scintillation counter. The following procedure details the preparatory steps.

1. Getter strips made of Whatman No. 1 filter paper are cut (8.8 cm x 3.7 cm) to fit into liquid scintillation vials. For photosynthesis experiments, a window (1 cm x 3.7 cm) is cut into each of the strips. The strips are dipped into concentrated Liquefluor solution, air dried for one hour in a hood, and inserted singly into the scintillation vials.
2. The uncapped vials are placed in a vacuum evaporator for 24 hours in the dark. They are stored, capped, in the dark until used.
3. Approximately four hours before use, the strips are wetted with 0.5 ml of sterile 1N NaOH, and the vials capped.

4. If required, 0.5g sand is added to the inner experimental tubes. These tubes are capped with a cotton insert and autoclaved for 30 minutes.
5. The labeled substrate is diluted, if necessary, and injected into the experimental tube using a sterile microliter or 1 ml syringe as required. Then the cells in the suspension appropriate to the particular experiment are injected into each experimental tube. This sequence is used to prevent transfer of the cells from tube to tube. If the liquid volume required is very small (<0.1 ml), the cells, nutrients, solution and labeled substrate are mixed together before addition to the experimental tubes. In either case, controls are injected with labeled substrate, nutrients and solution.
6. After each tube is injected, it is inserted uncapped into a strip-containing liquid scintillation vial which is subsequently capped and placed in the liquid scintillation counter. These vials are incubated therein at room temperature and counted as required.

In utilizing this technique for long term experiments, it was necessary to remove the inner vial every 300 hours and place it into a fresh outer vial containing a fresh fluor coated strip. This was necessary because counts contained on a fluor strip appeared to diminish somewhat with time. Thus, fluor

strips exposed to $^{14}CO_2$ and counted continuously showed a slow but continuous reduction in the recorded radioactivity such that an 8% loss was observed after 340 hours.

Bacterial enumerations within reaction vials were performed at the termination of a Buddemeyer-type experiment by examining the contents of the entire inner vial. A detergent solution was first prepared containing 30 µg/ml Linear Aklyl Sulfate (LAS, Witco Chem Corp, 90% activity) in Dilution Medium B. After removing the inner vial from the outer vial, 1.0 ml of the detergent solution was added to the silica base. The vial was capped and shaken vigorously for 30 seconds after which 0.5 ml was removed and added to 4.5 ml Dilution Medium B. Serial dilutions were performed and 0.1 ml aliquots spread plated onto Medium A with 1.5% agar. All plates were incubated at room temperature and counted after 48 hours.

The substrate generally used in the Buddemeyer experiments was the L isomer of ^{14}C-labeled sodium lactate. Radioactive solutions were prepared from uniformly labeled stocks at a specific radioactivity of 146.9 mCi/mmole and at a concentration of 678 nmoles/ml. For use in an experiment, the labeled lactate was adjusted to an appropriate radioactive concentration by diluting with inorganic dilution medium (Medium B). All solutions were stored at -10°C and thawed just prior to use. Periodic checks for contamination were

conducted using both the Lactate Minimal Medium (Medium A) combined with 1.5% agar and using Standard Methods Agar:

Medium C: Standard Methods Agar

Bacto-Yeast Extract	2.5 g
Bacto-Tryptone	5.0 g
Bacto-Dextrose (Glucose)	1.0 g
Bacto-Agar	15.0 g

Plates were incubated at 37°C and read at 48 hours.

3.1.1.2 Standardization of Repeated Addition Technique

A technique capable of extending the monitoring period of terrestrial/Martian interactions is repeated addition of ^{14}C-labeled substrate. Several experiments have been conducted to determine the optimal cell inoculum and the nutrient concentration, and influence of different amounts of suspending fluid on the effectiveness of repeated substrate additions. The principal bacterial species selected for this study was Isolate Ps-3. This organism, characterized as a Gram negative, obligately aerobic rod, capable of oxidative carbohydrate metabolism, has been tentatively identified as a Pseudomonas species. The production of a fluorescent green water soluble pigment on Standard Methods Agar (Medium C) suggests speciation as Pseudomonas flourescens. Other organisms used previously (1) in the repeated addition experiments were Azotobacter, and Isolate 8_0, a soil isolate. Since each culture responds similarly to the repeated addition technique in preliminary experiments, the Ps-3 Isolate was selected as representative for further refinement of the technique.

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3.1.1.2.1 Effect of Cell Inoculum on Response

In order to have a rapid method of enumerating the inoculum, a chart of optical density (420 nm) versus aerobic heterotrophic plate counts was prepared. Serial dilutions of cells grown in the Lactate Minimal Medium at 26°C on a Blue M Co. reciprocating shaker (setting = 15) were prepared. The optical density at 420 nm of these dilutions was determined in a Spectronic 20 against a Lactate Minimal Medium blank. The number of aerobic heterotrophs was determined by enumerating the colonies found on Lactate Minimal Medium spread plates after three days incubation at room temperature. A linear regression analysis of the data resulted in the following equation: $Y = (1.43X + 0.005)\ 10^9$, and the data have been plotted in Figure 1.

Previous studies with other bacteria (1) had shown that the length of the logarithmic phase of growth was extended as the number of cells inoculated was reduced. In an experiment to determine the optimal inoculum for this culture, sixteen double vial set-ups were inoculated in groups of four with approximately 10^3, 10^2, 10^1 and 10^0 cells respectively in 0.05 ml of Dilution Medium B. Each of these vials and three sterile control vials also received 0.05 ml containing 0.0076 mg of ^{14}C-lactate (0.01 μCi/vial). The data are presented in Figure 2.

Three additions were made at approximately 50 hour intervals beginning after 170 hours of growth. This initial stationary phase was prolonged (120 hours) so that any changes in the

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Figure 1

Relationship Between Ps-3 Cell Number and
Optical Density at 420 nm

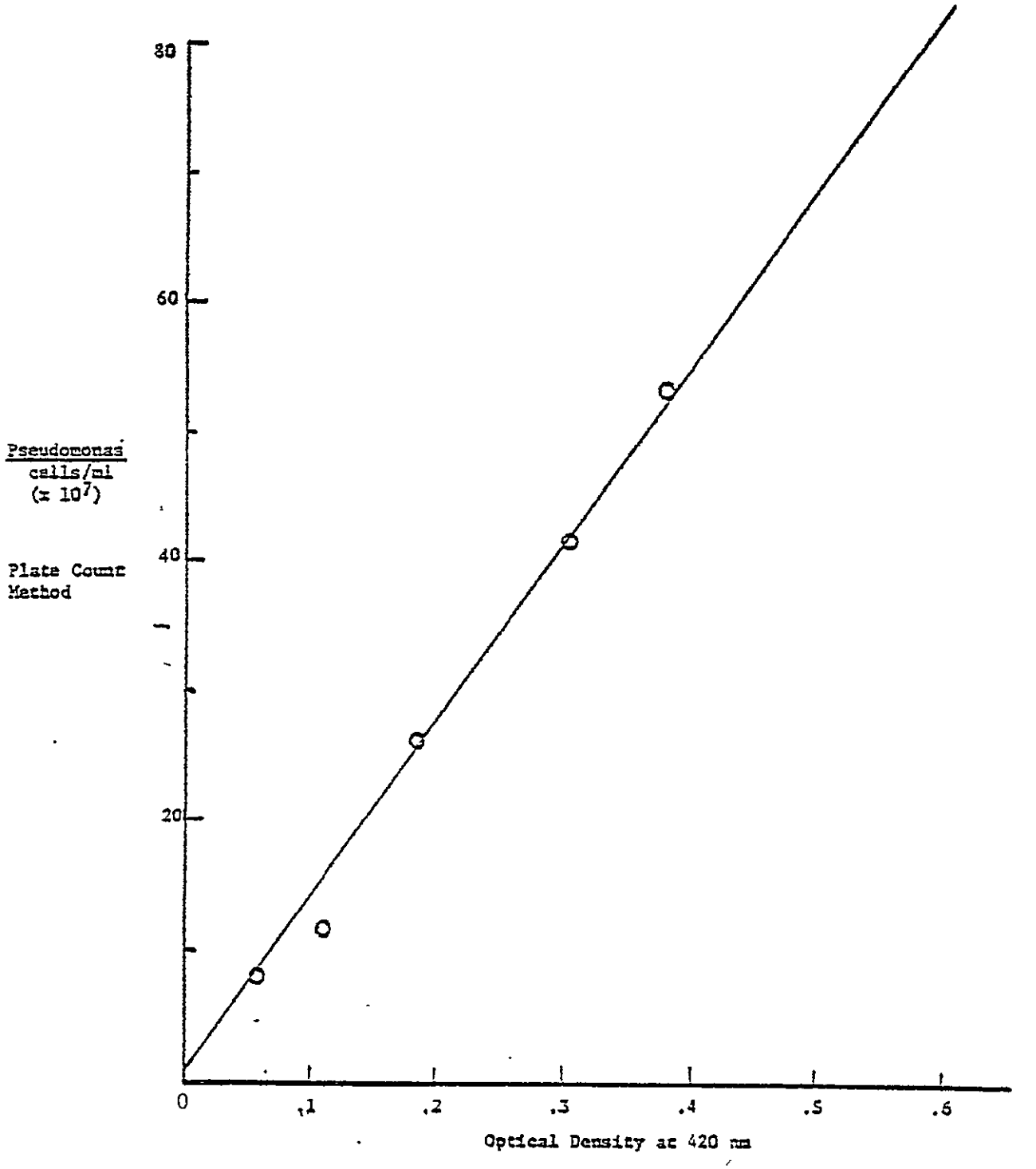

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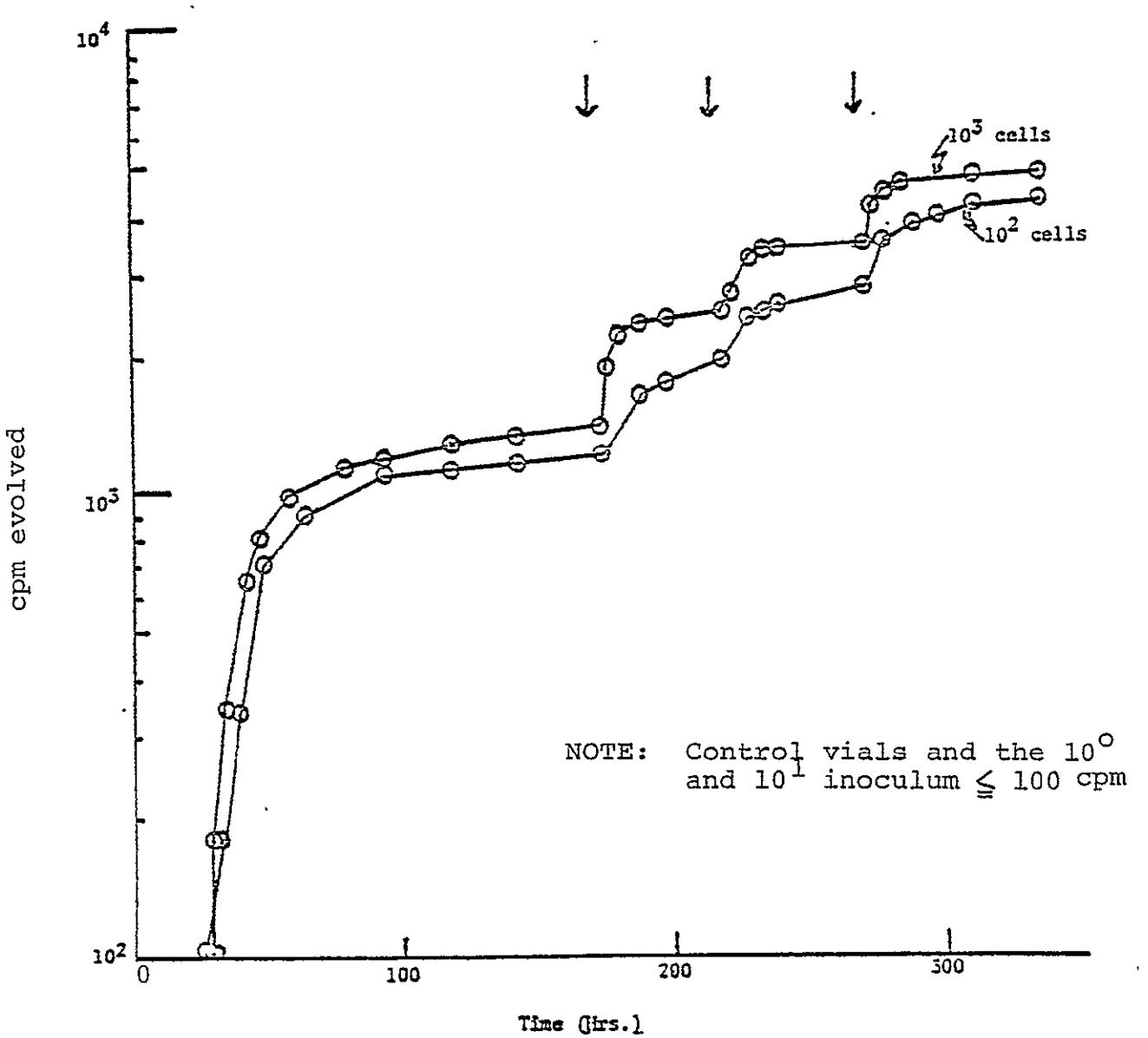
Figure 2

Response of Ps-3 to Repeated Substrate Additions



Four concentrations (10^0, 10^1, 10^2, 10^3 cells/vial) of Pseudomonas Ps-3 were mixed with 0.01 µCi of ^{14}C-lactate at appropriate intervals. Arrows indicate time of substrate additions.

rate of CO_2 evolution could be observed. The rate, however, remained relatively constant at approximately 2.5 cpm/hr. The 10^0 inoculum, as expected, gave results that paralleled the control. The 10^1 inoculum, shown by plate counts to contain $\approx$7.6 cells/vial, did not release more $^{14}CO_2$ than the control. Negative results were also obtained in a previous study (1) with this cell concentration, indicating either that this is a sub-critical population or that there is a variance between the calculated and delivered number of cells. $^{14}CO_2$ was evolved by both the 10^2 and 10^3 inocula. The kinetics of the reaction to the initial addition were the same for both the 10^2 (rate = 32 cpm/hr.) and 10^3 (rate = 37 cpm/hr.) inocula, although the actual amount of $^{14}CO_2$ evolved was less for the 10^2 inocula. However, the kinetics immediately following the second additions differed for these two inocula with the rate increasing to 64 cpm/hr. for the 10^3 inoculum but remaining constant at 37 for the 10^2 inoculum. Generally, the initial addition results in a typical growth curve response (lag, log and stationary phase), while subsequent additions result in immediate and rapid release of $^{14}CO_2$ followed by a stationary phase in which a reduced but measurable level of CO_2 release is maintained. Although a rapid increase in evolved $^{14}CO_2$ is observed upon the addition of label with the 10^2 inoculum, there is no definable entrance into the stationary phase. The differences between the 10^2 and 10^3 inoculum are not only the fewer number of cells, but also the substrate to cell ratio.

One explanation may be that more of the substrate is being utilized for cell biosynthesis in the 10^2 inoculum, since the "typical" pattern has appeared for inocula consisting of 10^3 or more cells in other studies (1).

Closer examination of these data shows the potential for several types of information. Calculation of the rates of evolution of $^{14}CO_2$ shows that the rate increases with each subsequent addition of substrate. For example, the rates of CO_2 evolution for the 10^3 inoculum were 37 cpm/hr. for the first addition, 64 cpm/hr. for the second, 86 cpm/hr. for the third, and 143 cpm/hr. for the fourth. Since the amount of substrate added is the same, this increased rate is indicative of an increased number of cells, showing that these cells are not only still capable of metabolism but also of cellular division, a condition highly desirable for the purpose of the BWS.

3.1.1.2.2 Effect of Nutrient Concentration on Response

In previous experiments, it had been noted that similar amounts of $^{14}CO_2$ were evolved from repeated additions of substrate. An experiment was undertaken to compare the kinetics of label release when different nutrient concentrations were added, and to determine the amount of nutrient required to change the response pattern. In one series of three vials, a constant amount of labeled nutrient (0.01 μCi in 0.0076 μg lactate) was used for each addition, while in a second series the amount added was increased

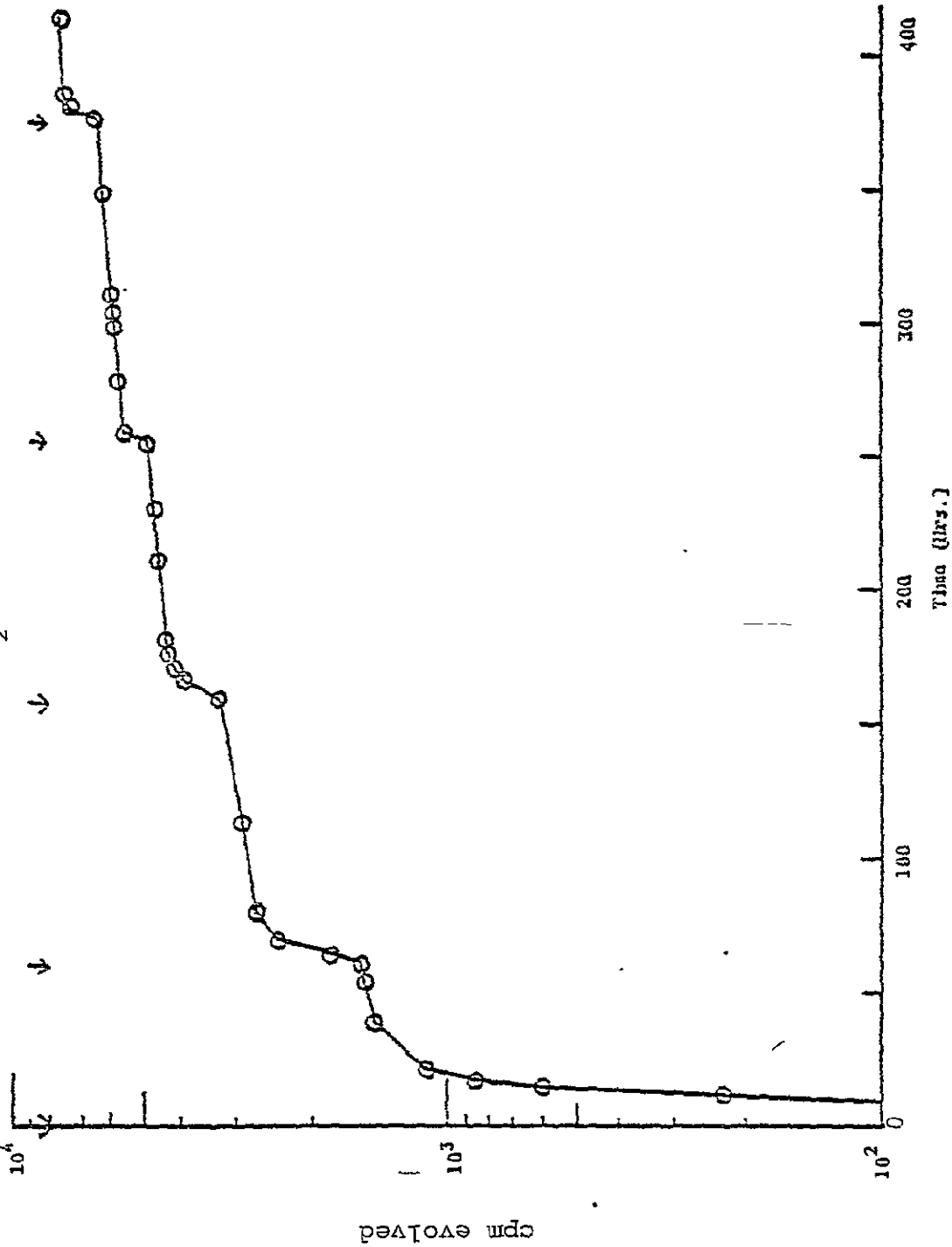
each time (0.01, 0.02, 0.04, 0.08, 0.02 μCi lactate). In both series the volume of the additions remained constant at 0.05 ml/addition.

The data from the repeated addition of 0.01 μCi (Figure 3) show that a rapid evolution of $^{14}CO_2$ occurred subsequent to each repeated addition of 0.01 μCi of lactate. Since each addition varied slightly because of normal dilution and pipetting variations, etc., the data have been standardized so that the responses may be compared (Table 1). Replicate amounts of the labeled substrate prepared for each addition were enumerated in the liquid scintillation counter so that the exact amount added at each addition would be known. Thus, it could be calculated that the amount of labeled substrate added in the second addition was only 0.77 times as much as was added in the first addition. Therefore, the response to the second addition, 1120 cpm, was recalculated to reflect this difference. This is shown in the sample calculation in Table 1. After the responses to the additions were recalculated to correspond to the actual amount of the label added, the response to each addition was compared to the response to the initial addition.

In the constant amount addition series, the responses to the second and third addition were 97 and 100% respectively, of the first, the fourth decreased to 82%, while the fifth returned to 90%. In the series with increasing concentrations

Figure 3

Effect of Repeated Substrate Additions on $^{14}CO_2$ Evolution by Ps-3



Repeated additions of 0.01 µCi ^{14}C-lactate were mixed with Ps-3 and subsequent evolution of $^{14}CO_2$ was monitored by standard procedures. Arrows indicate time of each addition.

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Table 1

Responses of the Ps-3 Isolate to Repeated Additions of Constant Amounts (0.01 μCi) of Labeled Substrate

Addition	Estimated Amount (μCi)	Measured Amt. Adjusted to 1st Addition as Base*	$^{14}CO_2$ Evolved (cpm)	$^{14}CO_2$ Calculated Evolved* (cpm)	Relative Response* (%)
1	0.01	1.00	1500	1500	100
2	0.01	0.77	1120	1455	97
3	0.01	0.77	1154	1499	100
4	0.01	0.77	951	1235	82
5	0.01	0.86	1159	1348	90

*Each subsequent value has been recalculated using the initial addition as the base.

Sample Calculation

$$\frac{^{14}CO_2 \text{ Evolved in Subsequent Addition}}{\text{Amount of Label Added/Initial Amount of Label Added}} = 1120/0.77 = 1455$$

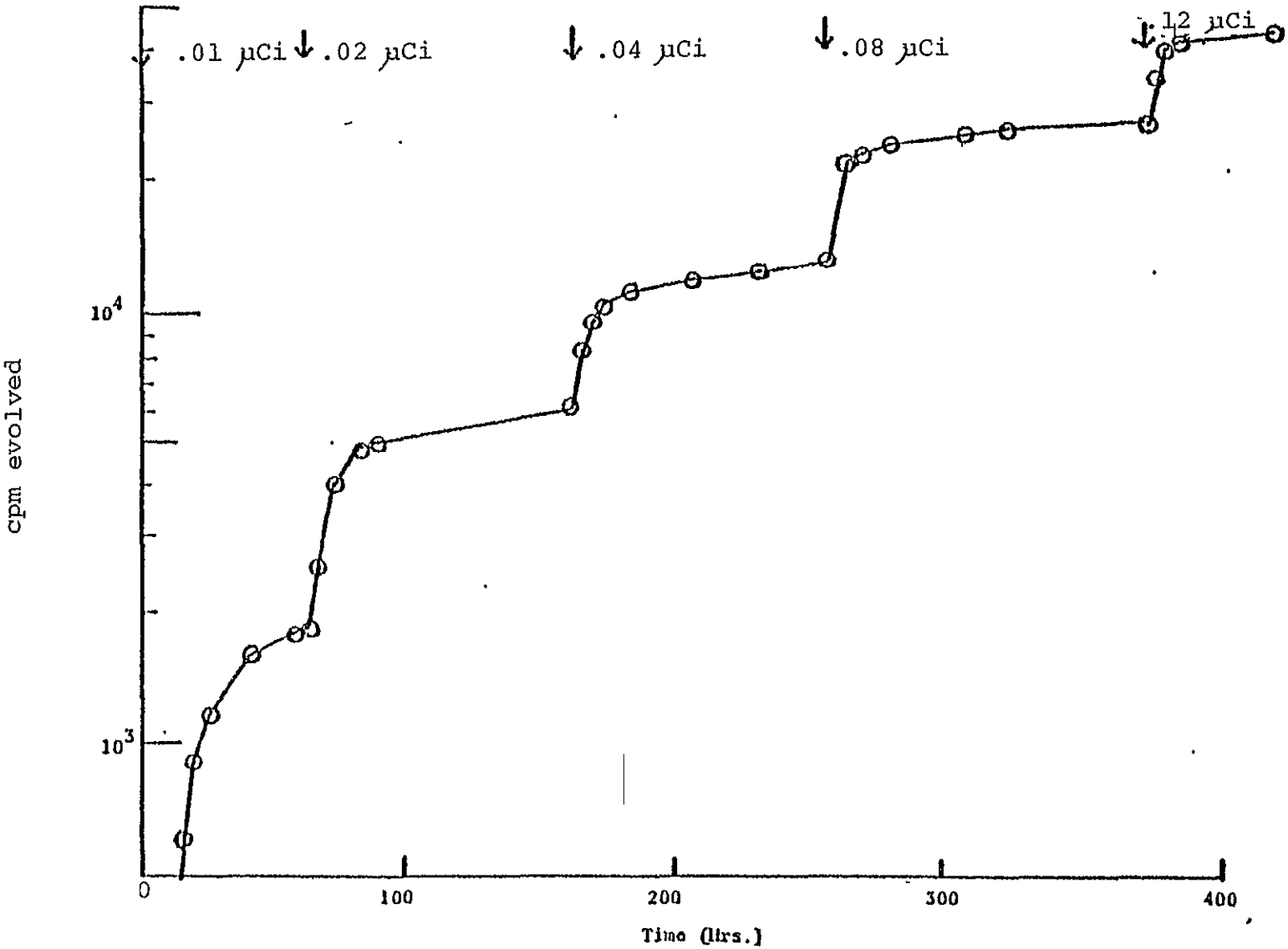
(Figure 4), none of the responses were less than 86% of the initial amount added (Table 2), and the $^{14}CO_2$ evolved was proportional to the amount of substrate added. This proportionality has been demonstrated for five additions spaced over a period of five hundred hours (20 days). Moreover, the rate of $^{14}CO_2$ evolution (Figure 4) increased with each increasing substrate concentration, showing that the population has increased.

In the constant amount series (Figure 3), the rate of $^{14}CO_2$ increases for each addition with the exception of the fourth addition where the rate decreases. This decrease in rate correlates with the decrease in the percent response (82%) and indicates that the population has been reduced or injured and/or that the substrate utilization pattern has shifted toward biosynthesis.

The results from the repeated addition of 0.01 μCi show that the maximum cpm evolved before the onset of a new plateau are in the range of 1500 cpm. At this level of counts, a reduction in metabolism (such as might be caused by Martian forms) of 20% would be only 300 cpm. Since the present experiment has shown that a higher substrate concentration can be used successfully for extended time periods, the concentration will be increased to 0.04 μCi in future experiments. Unless this increased activity level produces excessive noise, the statistical significance of the results should be improved.

Figure 4

Effect of Increasing Substrate Concentration
with Each Repeated Substrate Addition



^{14}C-lactate was added to Ps-3 in increasing concentrations, as indicated, and the reaction vials monitored for subsequent $^{14}CO_2$ evolution by standard procedures. Arrows indicate time of substrate addition.

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Table 2

Response of the Ps-3 Isolate to Repeated Additions of Increasing Amounts of Labeled Substrate

Addition	Estimated Amount (μCi)	Measured Amt. Adjusted to 1st Additions as Base*	$^{14}CO_2$ Evolved (cpm)	Relative Response* (%)
1	0.01	1.00	1656	100
2	0.02	1.86	3286	99
3	0.04	3.59	5776	87
4	0.08	6.88	12670	97
5	0.12	11.07	17098	86

*Each subsequent value has been recalculated using the initial addition as the base

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An additional observation resulting from this experiment is that the continued addition of the substrate in volumes of 0.05 ml results, after three additions, in a change from solid base to a liquid base. Since many additions are anticipated, and the liquid volume is so much greater than on Mars, the effect of adding the substrate in 0.01 ml increments will be evaluated. This will decrease the total liquid volume to which the Mars organism is exposed.

3.1.1.2.3 Effect of Liquid Volume on Response

These experiments were undertaken to determine whether the volume of liquid in which the nutrient material is added could be reduced from 0.05 ml to 0.01 ml without adverse effects. Eight replicate double vial set-ups were prepared and the bacteria inoculated in standard manner. The labeled substrate was then added in a 0.01 ml volume to three vials, a 0.05 ml volume to the other three vials, and a 0.05 ml volume to two sterile controls. Each volume contained a total of 0.04 μCi and 272 μmoles substrate unless otherwise specified. While the standard response occurred with the 0.05 ml additions, erratic responses, varying from 87 to 4723 cpm, were observed in the 0.01 ml vials. This experiment was repeated, and the variance noted with the 0.01 ml volume was verified. Possible explanations are the experimental error in adding such small volumes or that this volume was not sufficient to become evenly mixed with the added volume.

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The efficiency of the addition technique was determined to test the first explanation. Two concentrations of labeled material were prepared containing 0.04 μCi/0.01 ml and 0.04 μCi/0.05 ml, respectively. Thirty liquid scintillation vials containing 10 ml of the standard dioxane fluor were prepared. Ten were inoculated with 0.05 ml using a 0.01 ml pipet calibrated in 0.001 ml units, ten with 0.01 ml using a similar pipet, and ten with 0.01 ml using a 100 μl Hamilton syringe. After shaking, the vials were counted three times in the liquid scintillation counter and the results averaged.

The precision for the 0.05 ml addition was within 4.3%, while both the injected and pipetted 0.01 ml additions were between 6 and 7%. As one would expect, addition of the larger amount was more accurate. However, the 6 - 7% difference with the 0.01 ml volume was not large enough to account for the variance in response noted in the experiment. Thus, the conclusion that the 0.01 ml volume was unevenly dispersed within the vials with some bacteria in contact with higher concentrations of label appears the more likely.

As a result of the inoculation precision study, it was decided to use Hamilton syringes in future experiments rather than pipets because of ease of handling. Moreover, the smaller size makes it less likely that the syringe will contact the sides of the inner vial, causing carryover from vial to vial.

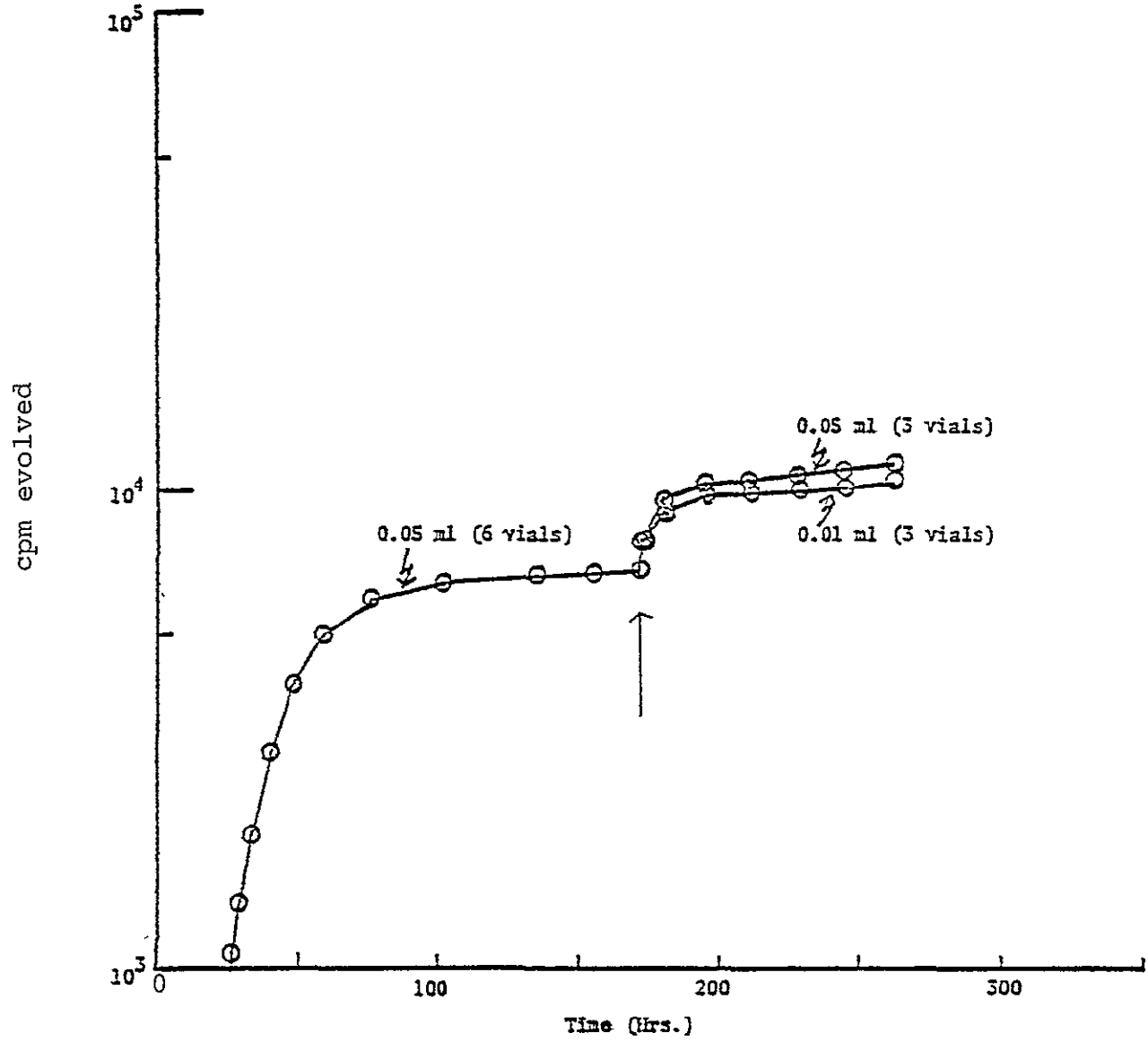
The possibility of using an initial addition of 0.05 ml

followed by subsequent additions of 0.01 ml was also explored. Consequently, six vials initially receiving 0.05 ml additions were divided into two groups, and 0.01 ml or 0.05 ml were added to three vials each. The $^{14}CO_2$ evolved was within 5% for each of these two groups (Figure 5) and the variance was within the range noted for other experiments. Based on these results, it was decided to apply the initial label addition in a volume of 0.05 ml, and subsequent additions will be in volumes of 0.01 ml.

Several sequential additions of either 0.01 ml or 0.05 ml to an initial 0.05 ml have also been studied (Figure 6). When several volumes of 0.01 ml are sequentially added to an initial 0.01 ml volume, responses of comparable magnitude are obtained from each addition up to 6 additions, after which little or no further response is obtained. The number of repeated additions producing typical responses is considerably more for the 0.05 ml volume. Thus, over a four month interval, 21 additions of 0.05 ml were made; in only one of these replicate vials was a reduced response obtained after the 20th addition. This establishes the feasibility of maintaining a viable experiment over a long period of time (see later section 5.0). A subsequent experiment revealed that the time elapsed after the first addition influenced whether a response was obtained from the second addition. Thus, a second 0.05 ml addition made at 600 hours gave only

Figure 5

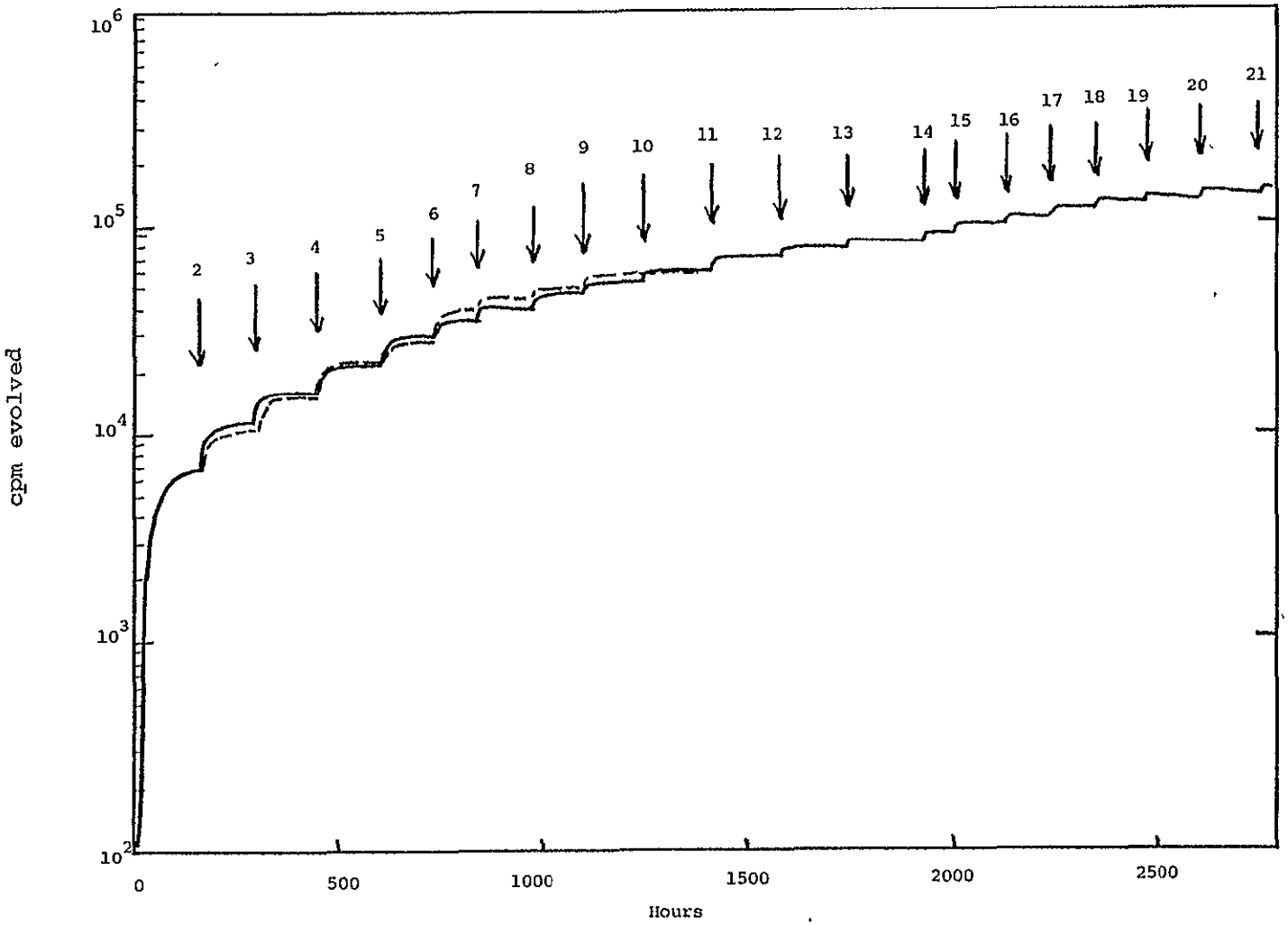
Effect of Volume on Repeated Addition Technique



^{14}C-lactate was added to Ps-3 in volumes of 0.01 ml or 0.05 ml after an initial injection of 0.05 ml. Both volumes contained 0.01 µCi substrate. Following each substrate addition, $^{14}CO_2$ evolution was monitored by standard procedures. Arrow indicates time of second addition.

Figure 6

Response from Repeated Additions of Substrate to Ps-3



The first addition of 0.04 μCi ^{14}C-lactate was added in a 0.05 ml volume to 0.05 ml Ps-3 containing 10^3 cells on a silica base. Subsequent substrate additions of 0.04 μCi were made in either a 0.01 ml volume (---) or a 0.05 ml volume (——). Each substrate addition is indicated with an arrow stating the addition number. Numerical data for the 0.05 ml additions are given in Table 21.

a 64% response whereas no response was noted using the 0.01 ml volume (Figure 7).

3.1.1.2.4 Kinetics of a Prolonged Response

Novitsky and Morita (8) recently showed that prolonged starvation of a bacterial culture produced changes in cell size; however, when fed, the cells grew with no noticeable lag period even after starvation periods as long as six weeks (1000 hours). This implies that prolonged periods can be used between substrate additions in order to maximize the duration of the assay period. This principle has been tested for applicability to the repeated addition technique.

Triplicate double vial set-ups plus controls were prepared and initial injections of 10^3 cells/.05 ml plus .01 μCi/.05 ml were made into each vial. The evolution of $^{14}CO_2$ was monitored without subsequent additions of substrate for a prolonged period (24 days) to permit observation of the kinetics of $^{14}CO_2$ evolution by moribund cells as measured by the Buddemeyer System. The initial response produced a sharp rise in radioactive gas evolved. The rate then leveled off after 80 hours and continued for up to 340 hours with a reduced rate of $^{14}CO_2$ evolution continuing during the stationary phase (Figure 7). Thereafter, a decrease of several hundred counts was noted (attributable to a reduction of the absorption capacity of the fluor - see standard procedures) after which no $^{14}CO_2$ was evolved for 200 hours. A second injection was

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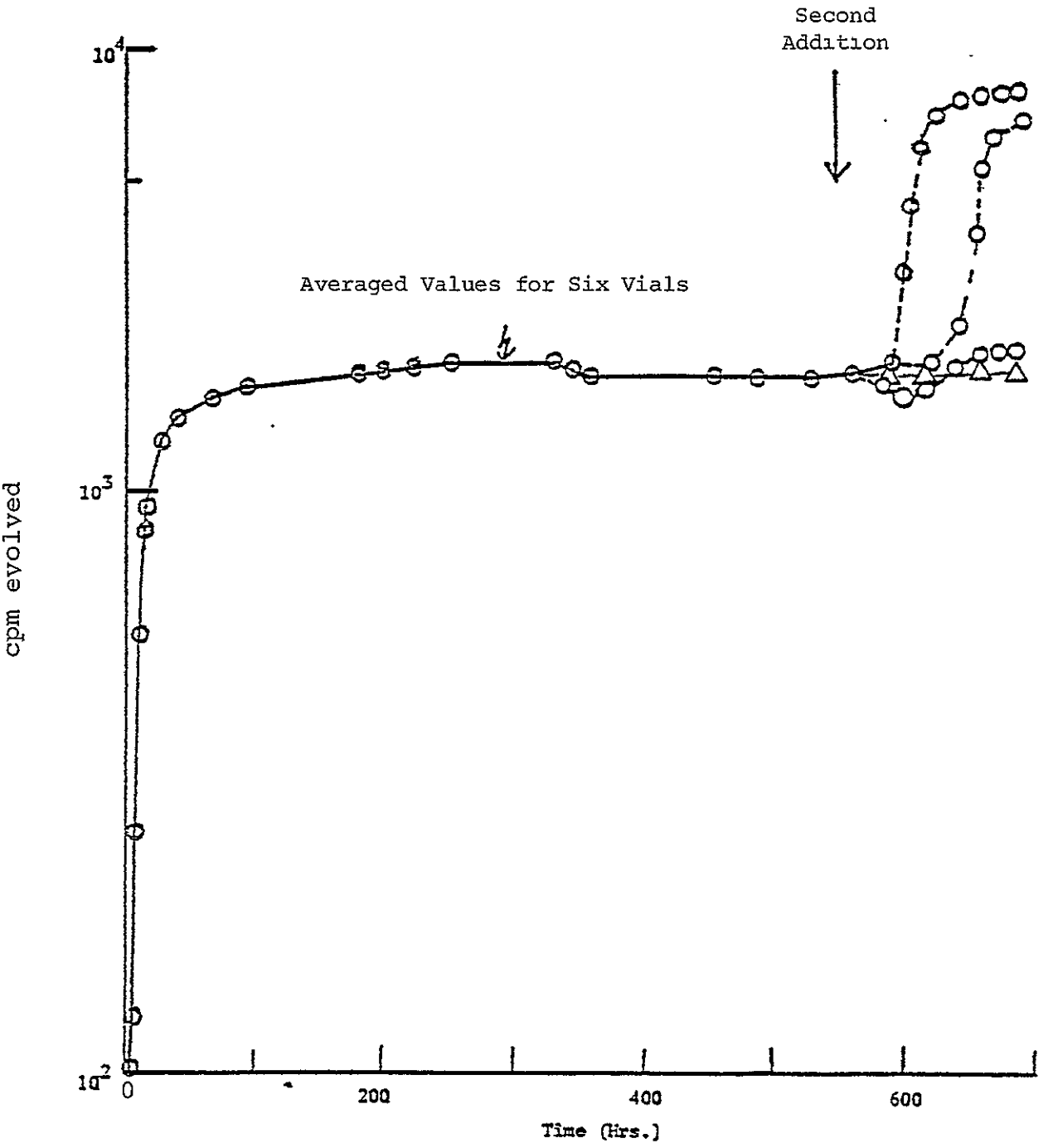
Figure 7

Effect of Volume on Response of Ps-3 to a Second Substrate Addition



Kinetics of $^{14}CO_2$ evolved from 0.01 µCi ^{14}C-lactate during a 24-day period, with a second addition at 576 hours. The initial injection volume into each of six vials was 0.05 ml. Second additions were in volumes of 0.05 ml (circles) or 0.01 ml (triangles). Data for second additions are presented for individual vials for the 0.05 ml volume where large variations occurred; for the 0.01 ml additions, little variation occurred, and the data have been averaged.

then made at 576 hours to determine whether any cells were viable. As discussed in the previous section, the response to the second addition has generally been 97 - 99% of the initial response. In this experiment, the $^{14}CO_2$ evolved was 2%, 78%, and 86% (three vials) of the initial response, indicating that cell damage had occurred within 576 hours or that a portion of the cells had become moribund. This is substantiated by the lag phases of 5 and 50 hours, respectively, in the two vials where metabolism occurred. Perhaps the reduced response reflects utilization of the radioactive substrate for structural repair rather than energy yielding metabolism with carbon dioxide as end product.

Since these results suggest that a change may occur at 340 hours, an additional experiment was conducted to establish the maximum interval between the first and second injection that would still yield a response equivalent to that from the first injection. Replicate vials were prepared and given first injections of 0.05 ml cells plus 0.05 ml substrate as above. Groups of triplicate vials received second injections immediately or at 42, 96, 193, 265, 360, or 500 hours. Controls were included as appropriate. In conjunction with the metabolic experiment, growth of a culture contained under conditions identical to those of the metabolic experiment was simultaneously followed by cell count to relate effectiveness of the second substrate addition with stage in the growth

cycle. The results showed that the maximum response (97 - 99%) from a second injection could be obtained after intervals of up to 265 hours after which the response diminished. At 360 and 500 hours, respectively, the responses were 95% and 80% of the initial response. The growth curve showed that maximal growth occurred within the first 100 hours. Stationary phase extended from approximately 100 to 265 hours after which the initial stages of death phase were observed. This experiment, combined with data from repeated additions (see previous section), suggests that extended monitoring periods are feasible using additions in approximately 8-10 day intervals.

3.1.1.3 Substrate Comparison

Having established the feasibility of using the repeated addition technique with lactate, the technique was next examined for applicability to other substrates. Those selected for comparison to lactate were formate, glycine, glycolate, and cellulose, all uniformly labeled with ^{14}C. Cellulose was included to establish the response from a substrate where metabolism was not anticipated by Isolate Ps-3. All other substrates were chosen because of their high probability of metabolism. For each incubation mixture, three injections of substrate were made at intervals of 168 hours. Each condition was examined in triplicate.

The results are summarized in Table 3. As shown, all four metabolizable substrates as well as a combination of

Table 3

Use of Repeated Addition Technique with Ps-3 and Various ^{14}C-Labeled Organic Substrates

Substrate	Specific Radioactivity (μCi/μmole)	Addition Number	Amount Added dpm	Amount Added nmoles	cpm evolved*	Relative % Evolved of Amount Added	Amount Evolved Relative to First Addition
UL-^{14}C-Lactate, Na salt	146.9 μCi/μmole	1	79,800	.247	6,091	7 6	1.00
		2	80,312	249	6,296	7.8	1.02
		3	83,967	260	6,376	7.6	1.00
^{14}C-Formate, Na salt	53 μCi/μmole	1	512,507	4.40	83,993	16.3	1.00
		2	479,100	4.11	60,462	12.6	0 77
		3	477,742	4 10	67,642	14.2	0.8?
UL-^{14}C-Glycine	80 0 μCi/μmole	1	69,530	.395	2,397	3 4	1.00
		2	69,344	394	5,210	7 5	2.17
		3	71,131	404	5,763	8 1	2.36
1-2-^{14}C-Glycolate, Ca salt	55 μCi/μmole	1	45,180	373	5,015	11.1	1.00
		2	44,202	.365	4,225	9.6	0 86
		3	40,531	.335	4,969	12.2	1.12
UL-^{14}C-Cellulose	10 μCi/mg	1	7,023		0		--
		2	7,877		0		--
Combined	--	1	702,977		101,320	14.4	1.00
		2	683,936		86,639	12.7	0.88
		3	675,771		71,370	10.6	0.73

*Counting Efficiency Unknown But Probably Between 5 and 15%.

Additions of 0 05 ml substrate were made at 168 hour intervals to each Buddemeyer Reaction Vial containing Ps-3 cells.

all five substrates gave a positive response from the initial addition. Cellulose, however, did not produce a response, as predicted. Repeated additions gave responses similar in magnitude to initial responses for all substrates except glycine where the response was enhanced by repeated additions. These results establish the practicality of utilizing this technique with a variety of substrates, used either singly or combined.

The effectiveness of utilization of each substrate cannot definitely be established because the counting efficiency for evolved gas in the method used can only be approximated between 5 and 15%. However, it appears that most substrates (except cellulose) were utilized to near completion. An approximation of the relative effectiveness can be estimated by calculating the percent of the added radioactivity in dpm evolved as cpm (to convert to actual percent efficiency, this number must be divided by the counting efficiency). If the counting efficiency can be assumed equal for all systems, it appears that formate and glycolate are more effectively utilized than glycine and lactate. However, because the total moles present in each assay varied among substrates, the conclusion must be considered tentative.

3.1.1.4 Applicability to a Nitrogen-Fixing Organism

The repeated addition technique was next examined for applicability to the nitrogen fixing organism, Azotobacter

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chroococcum. These cultures were grown either on lactate medium containing nitrate as nitrogen source (Medium A) or in a nitrogen fixing medium of the following composition:

Medium D: Nitrogen Fixing Medium

K_2HPO_4	0.4 g
Na_2MoO_4	trace
$FeCl_3$	trace
Lactate	0.1%
Purified Agar	1.5%
distilled water	to 1 liter
pH	7.2

Nitrogen fixing cultures were grown in 10 ml screw cap tubes at room temperature with shaking and were transferred weekly. The inoculum used for cultures grown on the nitrogen source (Medium A) was prepared essentially as described for heterotrophic bacteria using dilution Medium B. To prepare the inoculum from nitrogen fixing cultures grown in Medium D, 10 ml of Medium D was placed into a 13 x 150 mm screw cap tube and inoculated with a loop from a 1 week culture. This fresh culture was grown with shaking for 48 hours at room temperature. Each inoculum was then prepared by diluting the 48 hour culture to a concentration of 2×10^4 cells/ml according to optical density measurements at 420 nm. These dilutions were made using an inorganic medium of the following composition:

Medium E: Nitrogen Fixing Dilution Medium

K_2HPO_4	0.4 g
Na_2MoO_4	trace
$FeCl_3$	trace
Purified Agar	1.5%
distilled water	to 1 liter
pH	7.2

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Azotobacter cultures grown with or without nitrate in the growth medium were compared for response to the repeated addition technique using ^{14}C-labeled lactate as substrate. The results are presented in Table 4 and in Figure 8. As shown in Table 4, initial responses were somewhat greater for the culture grown in the nitrate free medium. In both cases, however, the magnitude of the second response was about 40% greater than the initial response. Responses following third and fourth additions were within 20% of that following second addition. Thus, the repeated addition technique can be used to monitor the metabolism of nitrogen fixing organisms. It is of interest, however, that both cultures showed a lag of several hours before a response to this initial substrate addition was obtained. For subsequent additions, this lag phase was reduced more for the culture grown in the absence of nitrate than for that grown in the presence of nitrate. It was also noted that once a response to any further substrate began, the rate of $^{14}CO_2$ evolution was consistently faster for cultures grown in the absence of nitrates.

One week after the final substrate addition in this experiment, incubation vials were enumerated to determine whether cell growth had occurred. For the triplicate vials with cultures grown in the presence of nitrate, one showed an increase from 10^3 initial cells to 10^5 cells/vial whereas the other two were no longer viable. Since one of these two

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Table 4

Repeated Addition Technique with
Azotobacter in Presence or Absence of Nitrate

Vial Contents	Addition Number	Lag (in Hrs) Before Onset of Response	Average cpm Evolved per Injection
Nitrate Free Medium and *Azotobacter* cells	1	22	5506
	2	1	7674
"	3	3	7088
"	4	4	8463
Nitrate Medium and *Azotobacter* cells	1	15	4138
	2	14	5908
"	3	6	7206**
"	4	4	6965**
Nitrate Free Medium Alone	1		0
	2		143
"	3		168
"	4		115
Nitrate Medium Alone	1		118
"	2		258
"	3		194
"	4		167

** Average of only 3 vials. The fourth vial did not respond to addition.

Injections of 0.05 ml ^{14}C-labeled lactate were made at 168 hour intervals and the resulting $^{14}CO_2$ evolution monitored by the Buddemeyer technique. For each injection, evolved cpm are given as the increase over the preceeding plateau. Averages are obtained from four replicate vials.

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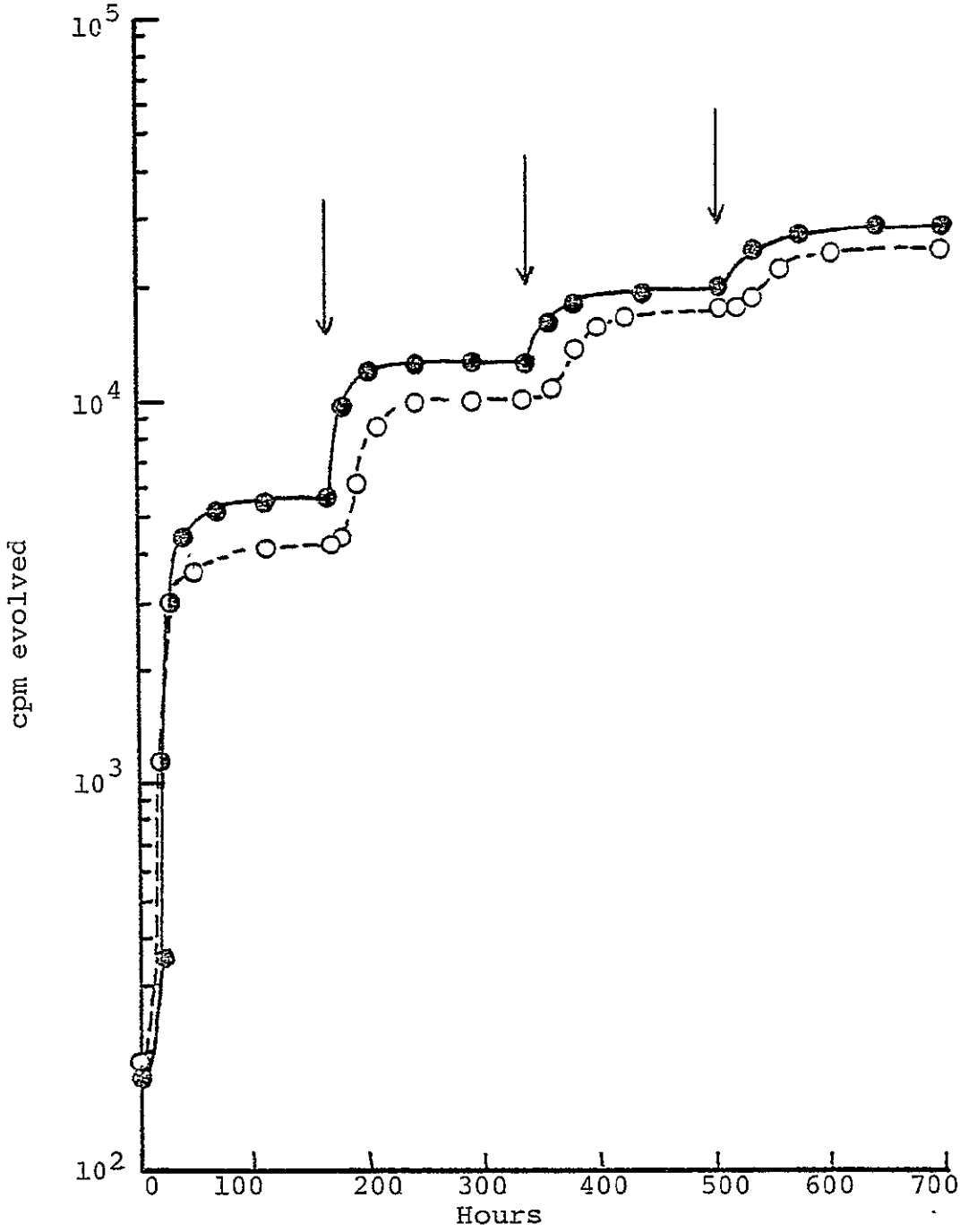
Figure 8

Response of Azotobacter to Repeated Addition Technique in Presence and Absence of Nitrate



Kinetics of $^{14}CO_2$ evolution from Azotobacter mixed with ^{14}C-lactate in the presence (O---O) and absence (●——●) of nitrate in the medium. Arrows indicate time of repeated substrate additions.

vials had also shown no metabolic response to the fourth substrate addition (see footnote to Table 4), this indicates that although growth probably occurred in all vials, the repeated addition technique probably could not have continued successfully longer than 4 - 5 weeks. On the other hand, the triplicate vials grown in the absence of nitrate all showed an increase of 10^3 to approximately 8 x 10^5 cells/vial. It is assumed that these organisms fixed atmospheric nitrogen to facilitate this growth although attempts to demonstrate concurrent nitrogen fixation by the acetylene reduction technique (9) were unsuccessful.

3.1.2 Photosynthesis

3.1.2.1 Standard Procedures

Photosynthetic activity was followed by monitoring the disappearance of $^{14}CO_2$ from the headspace above a cell culture. To perform these experiments, an aliquot of 0.1 ml of a test algal culture containing approximately 2 x 10^4 cells/ml was added to the bottom of a liquid scintillation vial. Initial experiments also contained 0.5 g silica base which was omitted from later experiments. All vials were then sealed with an airtight rubber septum through which gas aliquots could be injected or removed with a one ml gas-tight syringe equipped with a 23 gauge needle. Upon removal of a sample, the volume removed was replaced with an equivalent volume of sterile air.

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To initiate an experiment, the algal culture was sealed into a vial and one ml of $^{14}CO_2$ gas injected. The reaction mixture was incubated in a light box at an intensity of 120 footcandles of soft white tungsten light at room temperature (app. 26°C). At appropriate time intervals, one ml aliquots were removed through the septum and injected into a separate sealed vial containing 10 ml dioxane counting cocktail with 0.15 ml β-phenethylamine to trap carbon dioxide. The vial was shaken vigorously for 10 seconds and allowed to set undisturbed for 5 minutes prior to counting in a Beckman LS-200 liquid scintillation counter.

All photosynthesis experiments were performed with the blue green algae Nostoc muscorum (ATCC#27347). The cells were grown in a 125 ml cylindrical round bottom bottle containing 100 ml carbohydrate medium #10.

Medium F: Nostoc Carbohydrate Medium #10

$NaNO_3$	0.083g
K_2HPO_4	0.01 g
$MgSO_4 \cdot 7\ H_2O$	0.025g
$Na_2SiO_3 \cdot 9 H_2O$	0.027g
$CaCO_3$	0.040g
Ferric Citrate	0.0035g
Citric Acid	0.0035g
Distilled Water	1.0 L
pH	8.0

The cultures were incubated at room temperature in a light box at 30 to 40 footcandles of soft white tungsten light. Cultures were maintained by transfer of 0.1 ml to fresh culture medium at approximately one month intervals.

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To obtain an inoculum for a photosynthetic experiment, the cellular concentration of the culture was determined by optical density at 680 nm and the culture diluted to an appropriate concentration with growth medium. The relationship between optical density and concentration was established using six concentrations of Nostoc which were enumerated with a Petroff-Hauser counter. The contents of sixty-four squares were counted at each concentration. Linear regression analysis was used to determine the equation for the line, $Y = (3.039 \times 10^5) X + 9227$.

Unless otherwise specified, the $^{14}CO_2$ gas used in the photosynthetic experiments was generated in a separate gas-tight vial from 0.1 ml of a $NaH^{14}CO_3$ solution (10 µCi/ml; 10 µCi/1.04 µmoles) by acidifying with 50 µl of concentrated H_2SO_4. Previous experiments showed that this reaction was essentially complete within a few minutes. A one ml $^{14}CO_2$ aliquot was then removed from the sealed vial using a gas-tight syringe and injected into the photosynthetic incubation vial containing the algal culture. This $^{14}CO_2$ injection contained approximately 70,000 dpm distributed into the 19 ml headspace of the incubation vial. To ensure equality of the $^{14}CO_2$ injections into each incubation mixture, a separate $NaH^{14}CO_3$ reaction vial was used to supply $^{14}CO_2$ to each photosynthetic incubation mixture.

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3.1.2.2. Evaluation of Modified Buddemeyer System For Monitoring Photosynthesis

Prior to selecting the standard procedures discussed above for monitoring photosynthesis, an attempt was made to adapt the Buddemeyer system (7) as a standard procedure. One of the advantages of the Buddemeyer system is that it provides a means of obtaining relatively continuous measurements without disturbing or destroying the sample. However, several problems, discussed below, showed the system to be unsuitable.

3.1.2.2.1 Photo-Activation of Dioxane Fluor Strips

Because the dioxane fluor coated strip is known to be light sensitive, the effect of continued illumination was examined. In studies of heterotrophic metabolism, vials were incubated at room temperature in the dark liquid scintillation counter to facilitate continuous counting. However, photosynthetic algal studies required illumination in a light box. Because the cpm recorded for the first vial in a series was erratic, the possibility of photo-activation of the fluor strip was investigated.

Standard scintillation vials containing fluor coated strips were prepared. Duplicate vials were exposed to the light (50 footcandles) for ten minutes, monitored in the liquid scintillation counter, then placed in the dark and monitored at ten minute intervals. This was repeated three times at forty minute intervals. Control vials were main-

tained in the dark for the entire period, and monitored in parallel with the light exposed vials.

The results (Figure 9) show that the light activation does occur, but that the effect decreases rapidly upon return to the dark. The vials were re-exposed twice after forty minutes in the dark, and the effect observed to decrease each time. A control vial which had been kept in the dark for 120 minutes was exposed to light with similar results to the above test to establish that the decrease in effect was not due to the fluor aging. Further studies showed that this photo-activation effect is proportional to the light intensity; an illumination at 100 footcandles caused a proportionally higher effect than illumination at 20 footcandles.

3.2.2.2.2 Release of $^{14}CO_2$ by Fluor Strips

Since the fluor strips would be used in experiments to determine the uptake of $^{14}CO_2$ by algae, an experiment was designed to evaluate $^{14}CO_2$ release by the strips. Nine dioxane strips were first charged with $^{14}CO_2$. The strips were treated with fluor as described by Buddemeyer (7) and placed in liquid scintillation vials. To each inner vial was added 0.03 ml of sodium bicarbonate containing 0.03 μCi (100 mg $NaHCO_3$/ml). The addition of the 0.03 ml concentrated sulfuric acid caused release of the $^{14}CO_2$ which was gettered by the fluor strips. After ten minutes these inner vials were removed. The scintillation vials were placed in the counter

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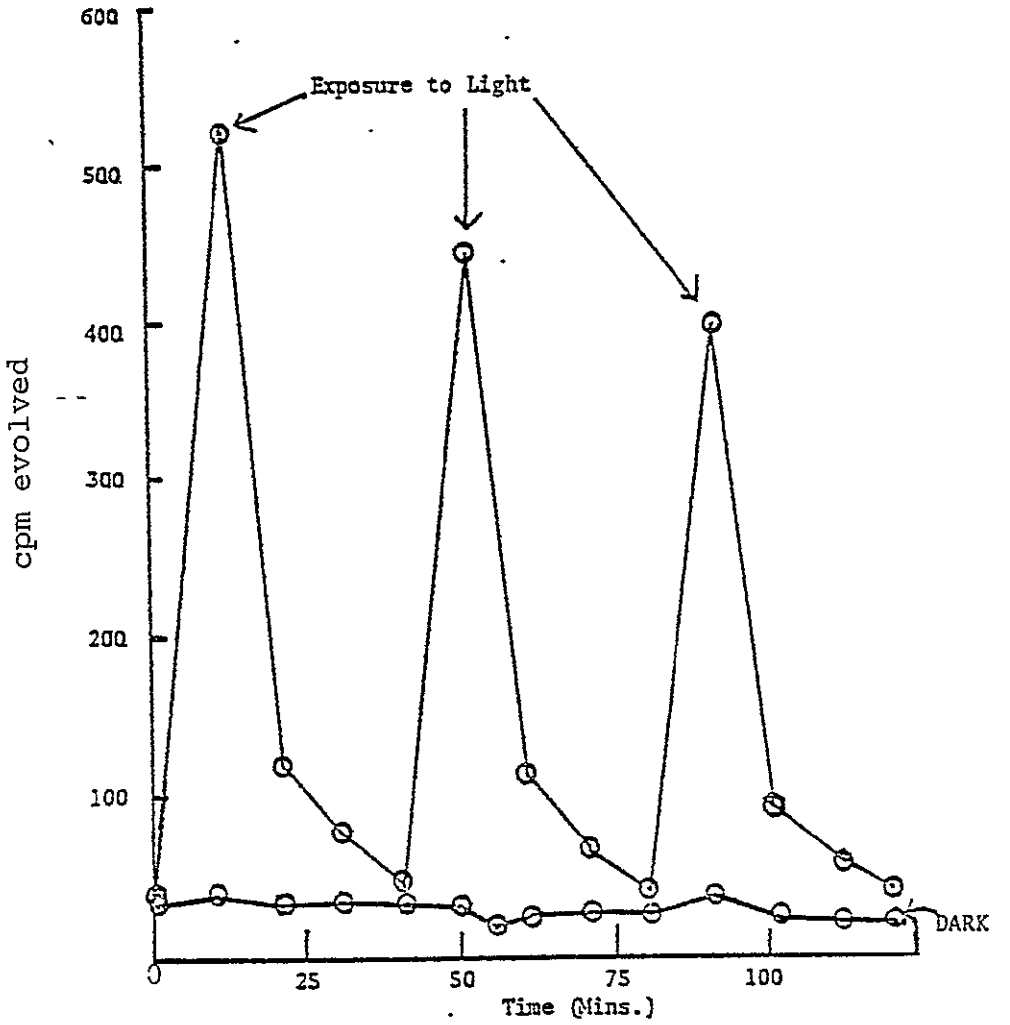
Figure 9

Photo-Activation of Dioxane Strip Fluors



Buddemeyer vials were exposed to the light at forty minute intervals and counted in a liquid scintillation vial. Control vials were maintained in the dark.

and the average cpm recorded was 6.4×10^4. Inner vials containing 2 ml Nostoc (4×10^3/ml) were then added to six other scintillation vials and sterile medium vials were added to the remaining three scintillation vials. Three of the Nostoc set-ups were incubated in the light, and three in the dark. After 48 hours, 15% of the $^{14}CO_2$ had been removed from the strips by the algae incubated in the light and 16% by the medium alone. These results mean that at least 10^4 cpm must have been released from the strips in the light or a deterioration of the fluor had occurred. For the algae incubated in the dark, however, an increase of 20% was observed (Table 5).

A second experiment was designed to further delineate this effect. In this experiment, five sets of vials were compared with each set performed in triplicate. All vials contained $^{14}CO_2$ charged dioxane fluor strips. Two sets also contained Nostoc cells at a higher concentration than in the previous experiment (2 ml of 5×10^5 cells/ml) and two additional sets contained medium alone. One of each of these sets was incubated in the light whereas the other two sets were incubated in the dark. The fifth control set received no media or cell additions and was incubated in the dark.

The results (Table 5) showed essentially no changes over the 48 hours incubation for the control strip incubated in the dark. However, both sets incubated in the light

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Table 5

Applicability of Buddemeyer Technique to Monitor *Nostoc* Photosynthesis

Experiment Number	Contents of Inner Vial	Incubation Condition	Percent Change in Radioactivity Level in 48 hours
1	10^3_3 cells and Media	Light; 26°C	-14.6
1	10^3 cells and Media	Dark ; 26°C	+19.7
1	Media Only	Light; 26°C	-16.2
2	10^6_6 cells and Media	Light; 26°C	-13.2
2	10^6 cells and Media	Dark ; 26°C	+ 9.4
2	Media Only	Light; 26°C	-16.2
2	Media Only	Dark ; 26°C	+12.3
2	Empty Vial	Dark ; 26°C	-0.62

showed a significant decrease in counts whereas both sets incubated in the dark showed an increase. These results, combined with those from the first experiment, suggest that the concentration of Nostoc has no effect on the phenomenon.

To determine whether $^{14}CO_2$ was leaving the strips in the light reactions, an additional experiment was conducted in which a $^{14}CO_2$ charged strip was incubated with an uncharged strip in light for 67 hours. Since media and cells were absent, no change was expected in the charged strip unless the uncharged strip could "pull" the reaction. At various intervals, the uncharged strip was removed, counted, and replaced with a fresh uncharged strip. At no time did an uncharged strip pick up counts or the charged strip lose counts, suggesting that $^{14}CO_2$ could not be "pulled" from the charged strip. However, since the charged strip did not lose counts in the presence of light, the results also suggest that light alone does not cause a deterioration of the fluors.

The reason for the observed changes was not pursued further. While the effects are not fully understood, sufficient data was obtained to indicate that the system was not well suited as a monitor for photosynthetic reactions.

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3.1.2.2.3 Effect of Buddemeyer System Components on Nostoc muscorum

Previous results (7) had shown that Nostoc is sensitive to one or more components in the Buddemeyer monitoring system. Several fluor systems were tested to see if a nontoxic system could be substituted, and the use of a semi-permeable membrane was investigated.

Standard double vials were prepared with Nostoc cells in the inner vials and various fluor carriers (toluene or dioxane) in both NaOH activated and unactivated forms on strips in the outer vials. Control vials contained paper strips moistened with sterile distilled water. Culture viability was evaluated visually since these cultures lose color on death. No difference was noted between the NaOH activated and unactivated vials. The toluene based fluor was less toxic than the dioxane, with cultures surviving more than four days before deterioration was observed. However, since long term photosynthetic experiments would exceed this limit, attempts were made to extend culture survival through the use of a semi-permeable membrane which would prevent transfer of toxic fumes to the inner vial. Silastic membrane squares were autoclaved and attached using rubber bands to inner vials containing 1.9×10^5 Nostoc cells. The experiment consisted of nine vials, three standard dioxane set-ups, three with the silastic membranes and

three containing untreated strips to serve as controls. A marked difference among the vials was apparent by the second day with visible disappearance of the algae in the vials exposed to the fluor, but not in the membrane covered or control vials. However, after four days, deterioration of the membrane covered cells was apparent, and the experiment was terminated. The average optical density of each set of three vials for the controls was 0.325, that for membrane covered vials was 0.182 and that for standard dioxane vials was 0.095, showing that the membrane does have a protective effect, but not enough to significantly extend the possible duration of photosynthetic experiments.

Since the membrane functions by selective permeability, its effect on the transfer of CO_2 was also evaluated by comparing the evolution of $^{14}CO_2$ by bacteria in membrane covered and uncovered vials. There was a reduction of 16% in the total amount of $^{14}CO_2$ released by the membrane protected cultures, but the kinetics of release were similar in both systems.

3.1.2.3 Application of Standard Procedures to Light/Dark Reactions

With the abandonment of the Buddemeyer technique to monitor photosynthesis and the implementation of the standard procedures described in an earlier section (3.1.2.1), several experiments were conducted to monitor Nostoc metabolism over

light and dark cycles. One typical experiment was conducted for fourteen days in which the first 24 hour incubation was in light and the next 6 days of incubation were in dark. The remaining four days of incubation were in either light or dark as specified in Table 6. Gas aliquots were removed from the headspace daily and counted. As shown in Table 6, the algal cells removed essentially all $^{14}CO_2$ from the headspace within 24 hours incubation in the light. When then placed in the dark, a small amount (~2%) of radioactivity was initially released indicating dark respiration. A gradual increase in evolved gas continued throughout the duration of the dark incubation. When again placed in the light after 7 days of incubation, no further changes occurred that could be attributed to photosynthetic activity although the cultures were visually still viable. Failure to observe photosynthetic activity during the second light interval may reflect the low levels of available carbon dioxide. Nonetheless, the results indicate the feasibility of monitoring algal photosynthesis followed by dark respiratory release by this technique. Photosynthetic activity was not observed in the medium alone controls; however, the decrease in radioactive gas that abruptly occurs upon placing these controls in the dark is unexplained.

Table 6

Effect of Light-Dark Sequences on Nostoc Photosynthetic Activity

Vial Contents	Light/Dark Sequence (Days)	Average cpm* per Vial on Day of Assay					
		Day 0	Day 1	Day 2	Day 7	Day 8	Day 11
0.5 ml algae in medium	Days 0→1=Light Days 2→7=Dark Days 8→11=Light	63728	24	689	1701	1689	1348
0.5 ml algae in medium	Days 0→1=Light Days 2→7=Dark Days 8→11=Dark	63728	31	870	1848	1620	1473
0.5 ml sterile media	Days 0→1=Light Days 2→7=Dark Days 8→11=Light	63728	69538	31902	19503	19766	16683
0.5 ml sterile media	Days 0→1=Light Days 2→7=Dark Days 8→11=Dark	63728	57845	29408	18951	14863	13675

*Reaction vials containing algae were performed in triplicate; those containing sterile medium were singlets.

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3.1.3 Elementary Ecosystems

Having established techniques to monitor either heterotrophic or photosynthetic metabolism individually, some preliminary experiments were conducted in an attempt to monitor both types of metabolism contained within one incubation chamber. Such experiments are prerequisite to the development of an ecosystem approach for monitoring vital processes.

The principal upon which the ecosystem concept has been approached consists of mixing cultures of the heterotrophic isolate Ps-3 and the photosynthetic Nostoc with ^{14}C-labeled lactate. Duplicate cultures are incubated at room temperature either in light or dark. The assumption is that as labeled lactate is consumed by the heterotroph, $^{14}CO_2$ will be evolved that could then be utilized by the phototroph. Theoretically, only heterotrophic activity will occur in the dark (with minor dark fixation of $^{14}CO_2$), but both heterotrophic and photosynthetic activity will occur in the light. Thus, differences in the amount of carbon dioxide present between the light and dark ecosystems presumably result almost completely from photosynthetic activity.

Although many possible monitoring systems were screened for use with the ecosystem, the one finally selected was identical to that used for photosynthetic experiments (see previous section) whereby gas samples were removed at intervals from the headspace above the incubation mixture and counted to

determine the amount of radioactive gas. Incubation mixtures contained 10^3 Ps-3 cells in a 0.1 ml volume, 10^3 Nostoc cells in a 0.1 ml volume, and 1.8 ml liquid medium G of the following composition:

Medium G: Combination Medium for Elementary Ecosystems	
K_2PO_4	0.04 g
$MgSO_3 \cdot 7H_2O$	0.025 g
$Na_2SiO_3 \cdot 9H_2O$	0.027 g
$FeCl_3$	0.001 g
$CaCO_3$	0.02 g
distilled water	to 1 liter
pH	7.4

Each inoculum was prepared as described previously for studies with each culture separately. Each reaction was initiated by the addition of 0.01 ml containing 0.32 µCi (7×10^5 dpm) of the ^{14}C-labeled lactate to the incubation mixture. (It should be noted that other experiments substituting 0.5 g silica base for the 1.8 ml Medium G gave similar results to those reported below).

The results of a typical experiment are shown in Figure 10. As shown, both algae and bacteria are capable of metabolizing lactate in the dark, although bacteria are considerably more efficient than the algae, as expected. When both cultures are mixed in the dark, lactate utilization is essentially identical to the summation of its utilization by the individual cultures in the dark. In the light, bacterial metabolism of lactate is essentially the same as in the dark. Algae, however, do not utilize lactate in the light, presumably

because they prefer photosynthetic fixation of atmospheric carbon dioxide. When algae and bacteria are combined in the light, radioactive gas initially appears in the headspace but then quickly disappears. This result suggests that the heterotrophic cultures are generating $^{14}CO_2$ which is then consumed by the photosynthetic cultures, thereby demonstrating that a carbon cycle can be established in an elementary ecosystem.

That the bacterial cultures increase in number over the three day duration of the elementary ecosystem was demonstrated in a similar experiment whereby 7×10^5 cells were enumerated at the end of the experiment, an increase of almost three orders of magnitude. This increase, combined with the results from the control bacterial culture in the absence of algae, suggests that bacteria in the presence of algae also evolve $^{14}CO_2$ continuously throughout the experiment. However, at no time was the carbon dioxide end product from heterotrophic metabolism allowed to accumulate in the headspace (Figure 10). This suggests that carbon dioxide was limiting for the phototrophic activity and that each generated $^{14}CO_2$ molecule was incorporated immediately upon becoming available once activity had been initiated. In future efforts, it would be of interest to test various combinations of algae and bacterial cultures under various environmental conditions to demonstrate repeated cyclical evolution and utilization of carbon dioxide. This may be

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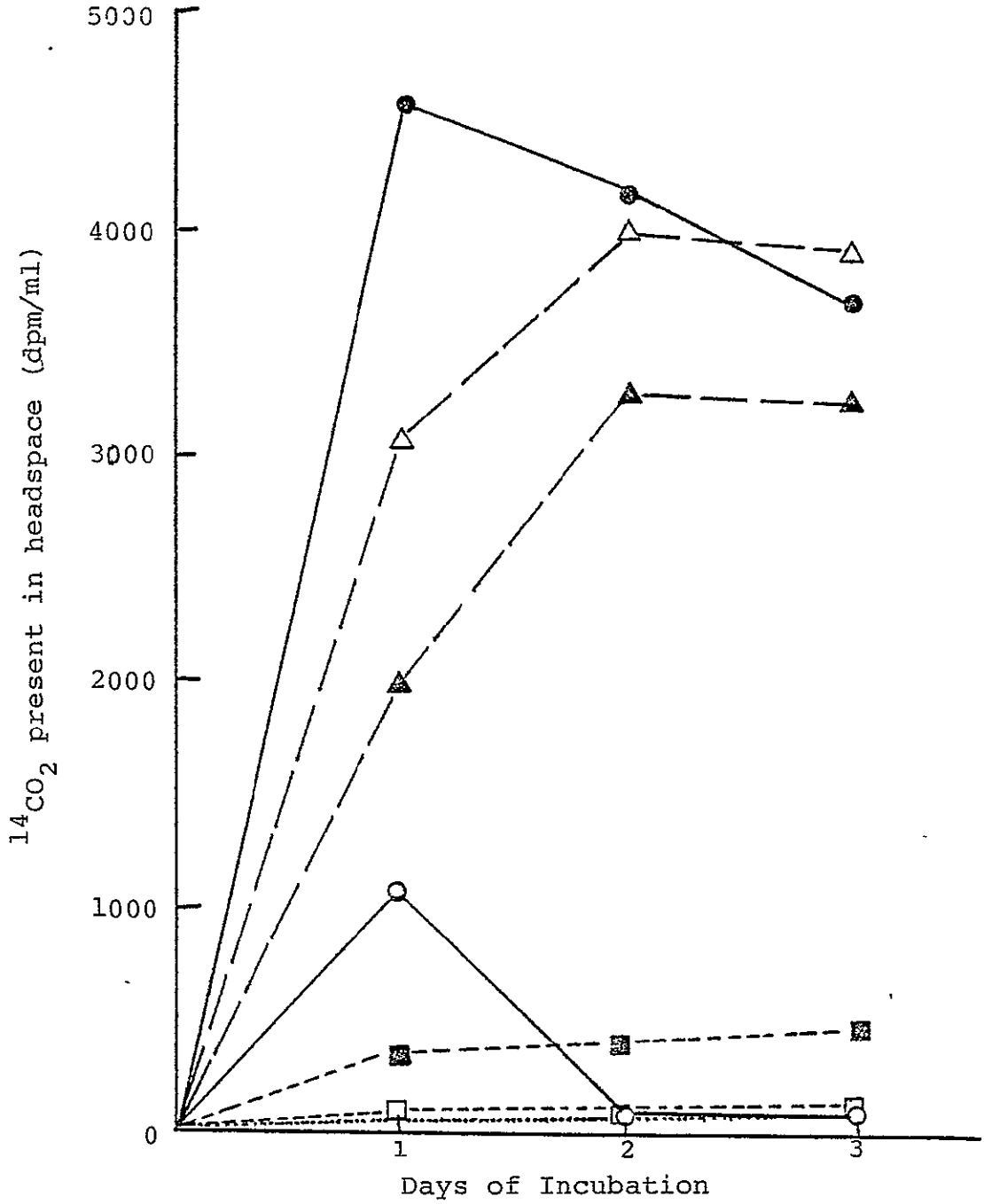
Figure 10

$^{14}CO_2$ Appearance and Disappearance in Elementary Ecosystem



The elementary ecosystem contains ^{14}C-lactate, the algae Nostoc, and the bacterial isolate Ps-3. $^{14}CO_2$ metabolism was followed for reaction vials incubated at 26°C in the light (open symbols) or dark (closed symbols). Reactions were conducted with Nostoc alone (----), Ps-3 alone (— —) and with Nostoc and Ps-3 combined (———). A control with sterile medium (.....) is also shown).

possible if an algae culture, or algal concentration, can be identified that requires a critical molar concentration of carbon dioxide before photosynthetic activity can be initiated. Alternatively, excess bacterial numbers could be utilized to supply a large excess of $^{14}CO_2$. Finally, environmental conditions using cyclic light and dark periods of varying duration or intensity could be explored to demonstrate a repeated carbon cycle in the elementary ecosystem.

3.2 Hardy Organism Techniques

The hardy organism system depends on utilization of a terrestrial organism capable of metabolism under conditions approaching those on Mars as closely as possible. It is assumed that such conditions will impose a minimum stress on any existent Mars organisms, thereby encouraging expression of Mars metabolism on living terrestrial organisms. Thus, this system tests effects of a viable Mars sample on terrestrial metabolism and constitutes a vital portion of the Basic Warning System.

In a previous report (1), several organisms were examined as candidates for use in the hardy organism system. Each was subjected to conditions approximating Mars environmental conditions to determine tolerance to the particular stress. The organism selected for further development of the hardy organism system was Rhodospirillum rubrum which could maintain growth and/or metabolism under minimal

moisture, at reduced temperatures, under a variety of atmospheres, and in the presence of ultraviolet light equivalent to 1/15th of that estimated for the surface of Mars. The ability of this organism to withstand each stress individually is summarized in Table 7 along with its ability to withstand selected stress combinations. Although each stress reduced the growth or metabolic rate of R. rubrum, sufficient growth or metabolism was nonetheless maintained to render this organism appropriate for the hardy organism technique.

In the continued development of the hardy organism component of the BWS, a chamber in which Mars conditions could be approximated has been developed to examine R. rubrum under a combination of the stresses such as those described above. This chamber permits optimization of conditions most closely approximating Mars and still permitting sufficient R. rubrum metabolism to serve as a monitor for the BWS. The standard procedures used for R. rubrum and the development of the Mars chamber are described in this section.

3.2.1 Standard Culture Methods

Cultures of Rhodospirillum rubrum were obtained from the American Type Culture Collection (#1170) and grown on either of two complete enriched medium of the following compositions:

Table 7

Tolerance of Rhodospirillum rubrum to Individual and Combined Stresses*

Parameter Tested	Constant Test Conditions	Variables	Result		
			Numerical		Units
Limited Moisture	Anaerobic 26°C Extreme Dessication	Control 100 µl H_2O 10 µl H_2O 1 µl H_2O	100 82 48 11		Percent Substrate Utilization/ 24 hrs.
Temperature		Anaerobic, 26°C Aerobic, 26°C Aerobic, 15°C Aerobic, 10°C Aerobic, 5°C	++++ ++++ + - -		Amount red color/ 48 hrs. (Qualitative)
			25 hrs.	100 hrs.	
Atmosphere; Light	15°C	Anaerobic, Light Aerobic, Light Aerobic, Dark Anaerobic, Dark	240,000 110,000 30,000 15,000	280,000 320,000 400,000 350,000	cpm evolved
UV Exposure	26°C ±UV Exposure, then incubate 3 days under fluorescent lighting	Anaerobic Aerobic Aerobic, UV	10^{10} 10^{8} 10^{6}		cells/ml
UV Exposure	15°C Light/Dark = 16/8 hrs. Anaerobic	-UV +UV	3200 2200		cpm evolved/ 150 hrs.
Combined Stresses	15°C Light/Dark = 16/8 hrs. Anaerobic	100 µl H_2O 1 µl H_2O	1000 250		cpm evolved/ 100 hrs.

*Data Summarized from Reference 1

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Medium H: Rhodospirillum Complete Medium
(Van Niel's Yeast Extract Medium)

K_2HPO_4	1.0 g
$MgSO_4$	0.5 g
Yeast Extract	5.0 g
Tap Water	1.0 L
pH	7.0 - 7.2

Medium I: Rhodospirillum Complete Defined Medium

KH_2PO_4	0.06%
K_2HPO_4	0.09%
$(NH_2)^4$ SO_4	0.1 %
$MgSO_4$ $7H_2O$	0.02%
$CaCl_2$ $2H_2O$	0.008%
$FeSO_4$ $7H_2O$	0.001%
DL-Malic acid	0.6 %
EDTA	0.002%
Biotin	1.5 µg/100 ml
$Cu(NO)_2$ $3H_2O$	7 ug/100 ml
H_3BO_3	280 µg/100 ml
$MnSO_4$ $4H_2O$	210 µg/100 ml
Na_2MoO $2H_2O$	75 µg/100 ml
$ZnSO_4$ $7H_2O$	24 µg/100 ml
pH	6.8

Cultures were grown without shaking in 10 ml of Medium H covered with a 5 ml overlay of mineral oil to limit exposure to oxygen. These cultures were placed in a light box at 125 footcandles tungsten light at room temperature (app. 26°C) and growth followed visually as described in a previous report (1). Development of a distinct red color is indicative of active growth and high numbers.

To facilitate adaptation of this organism to the BWS, a minimal growth medium was also developed for R. rubrum of the following composition:

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Medium J: Rhodospirillum Minimal Growth Medium

Sodium Lactate	0.1% (W/V)
$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Biotin	Trace
distilled water	to volume
pH	7.2 to 7.4

Cultures were grown on the minimal medium in the light with an oil overlay as described above for growth in complete medium. The minimal medium was designed to permit photoheterotrophic growth using lactate as carbon source and sulfate as electron donor. It should be noted that Medium J is identical to Medium A with a trace of biotin added, since biotin is a known requirement for photosynthetic activity by non-sulfur purple bacteria. In Medium H, biotin additions were unnecessary since biotin is a constituent of yeast extract.

Plate counts for cultures grown on Medium H, I, or J were performed by diluting Rhodospirillum rubrum cultures with Medium B and inoculating pour plates of the same growth medium containing 1.5% agar. Plates were incubated in an anaerobic jar under an atmosphere of hydrogen and carbon dioxide (generated from a BBL GasPak) in light as above. A BBL anaerobic indicator was added to the jar to ensure continued anaerobiosis. Plates were counted after three weeks' growth at room temperature.

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3.2.2 Re-Furbishment of Mars Box

The hardy organism system must be developed in a chamber in which a range of Mars-like environmental conditions can be studied, so that the operating conditions for the BWS can be defined. The available chamber that could be adapted for this purpose had been constructed for a previous project (Contract No. NASW-5360, 1969) and has been overhauled for use in this project. Basically, it consists of a rectangular vacuum chamber approximately 20" x 20" x 15" with attached probes and meters to provide control of environmental parameters. A sketch of the apparatus is shown in Figure 11, and a description of the components are provided below:

1. Environmental Chamber - Insulated stainless steel, containing necessary heating coils and cooling lines, with a 1' x 1' x 1' inner working area as well as a fully opening door with a 6" diameter viewing port. In preparation for this part of the study, the 1½" feedthrough port for electrical and mechanical access was complete refurbished. All of the glass vacuum connections have been vacuum tested, and the tubing and wiring replaced. The following connections are served by this feedthrough:

   a. two temperature probes

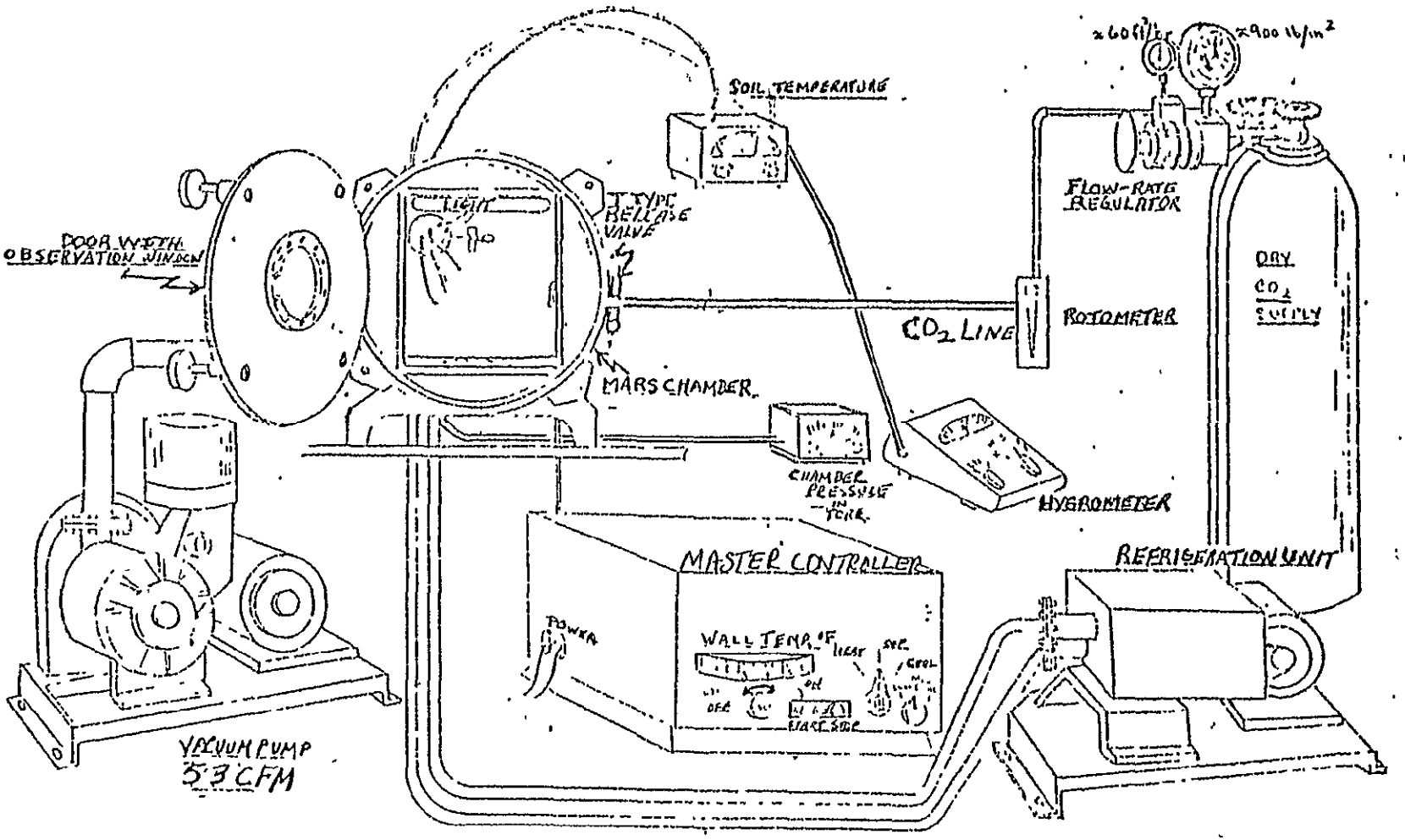
   b. one hygrometer probe

   c. one interior 110 volt socket with on/off switch

   d. one ultraviolet light

Figure 11

Diagram of the Mars Experimental Facility



APPARATUS FOR EXPERIMENTATION DONE UNDER SIMULATED MARTIAN CONDITIONS

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e. one fluorescent light

f. two tubes for transport of gas or liquids

2. Vacuum Pump - A large capacity (53 C.F.M.), high vacuum, mechanical pump (Welch 1398M) has been provided both to remove terrestrial gases and to maintain Mars atmospheric pressure (6 TORR). The pump was overhauled by Metra, Inc., College Park, Maryland. During operation a vacuum of 10^3 TORR is drawn on the chamber to remove terrestrial gases, and an atmospheric pressure of 6 TORR then provided by balancing gas inflow against pumping rate.

3. Control Instrumentation

a. Barber Coleman Capacitrol Controller (272P). This is the major control panel, containing the on/off switches for the vacuum pump, the lighting, the electrical outlet, and the heating/cooling system. The temperature range extends from $-100^{\circ}F$ to $300^{\circ}F$ and is measured by means of a thermistor inserted through the vacuum chamber wall.

b. Lighting. Three lighting options are available: no illumination, fluorescent (General Electric, F8T5) or ultraviolet (General Electric G8T5). The ultraviolet light provides 0.14 watts/sq. ft. of 2537Å at a distance of one foot ($\approx$1/3 Mars UV flux). The tubes are 12" long, providing illumination throughout the chamber. They are affixed to the roof of the chamber using 10-108

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Moburn fixtures, but can be wired to provide horizontal lighting if necessary.

c. Flowrator Meters - Atmospheric pressure is regulated through two rotometers (Fisher and Porter Co., Warminster, Pa., Lab Crest Type 10A1460) designed to control the rate of flow in the range 400 to 40,000 cm^3/minSTP. The rotometers have been connected in parallel to prevent damage to the reduced flow meter. Both are connected to a dry, O_2-free tank of carbon dioxide through a two-stage CO_2 regulator.

4. Monitoring Instrumentation

a. Temperature Gauge - The Simpson Model 389 gauge is connected to the chamber through two thermistor probes. One is attached to the probe from the Barber Coleman Controller, permitting comparison of the two meters. The other probe may be placed in a duplicate sample to measure sample temperature. This probe can be attached to a Strip-Chart recorder for continuous measurement.

b. Vacuum Gauge - Two vacuum gauges are used to span the measurement range. The Hastings Vacuum Gauge Model VT-452 (0.1 to 10 mm) is connected to the chamber through a DV-4D gauge tube. The Venco Gauge, Model DO-91 (1-1,000 microns) is connected through 2110 gauge tube. Both of these are connected to the pump chamber fitting.

c. Moisture Gauge - A Panametrics Model 1000 Hygrometer (Waltham, Mass.) has been attached to the vacuum feed-through from dew/frost points of +20°C to -110°C

and can be attached to a strip-chart recorder. The probe was returned to Panametrics for recalibration, and a new calibration curve was received.

3.2.3. Procedures for Metabolic Experiments and Preliminary Studies for Adaptation to Harsh Environmental Conditions

Experiments designed to monitor the metabolic response of Rhodospirillum rubrum under an anaerobic atmosphere utilized ^{14}C-labeled lactate and the Buddemeyer double vial system (7) described previously for monitoring heterotrophic metabolism. The R. rubrum inoculum was prepared by diluting a standard culture grown on Minimal Medium J in dilution Medium K of the following composition:

Medium K: Rhodospirillum Dilution Medium

$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Biotin	Trace
Distilled Water	to volume
pH	7.2 to 7.4

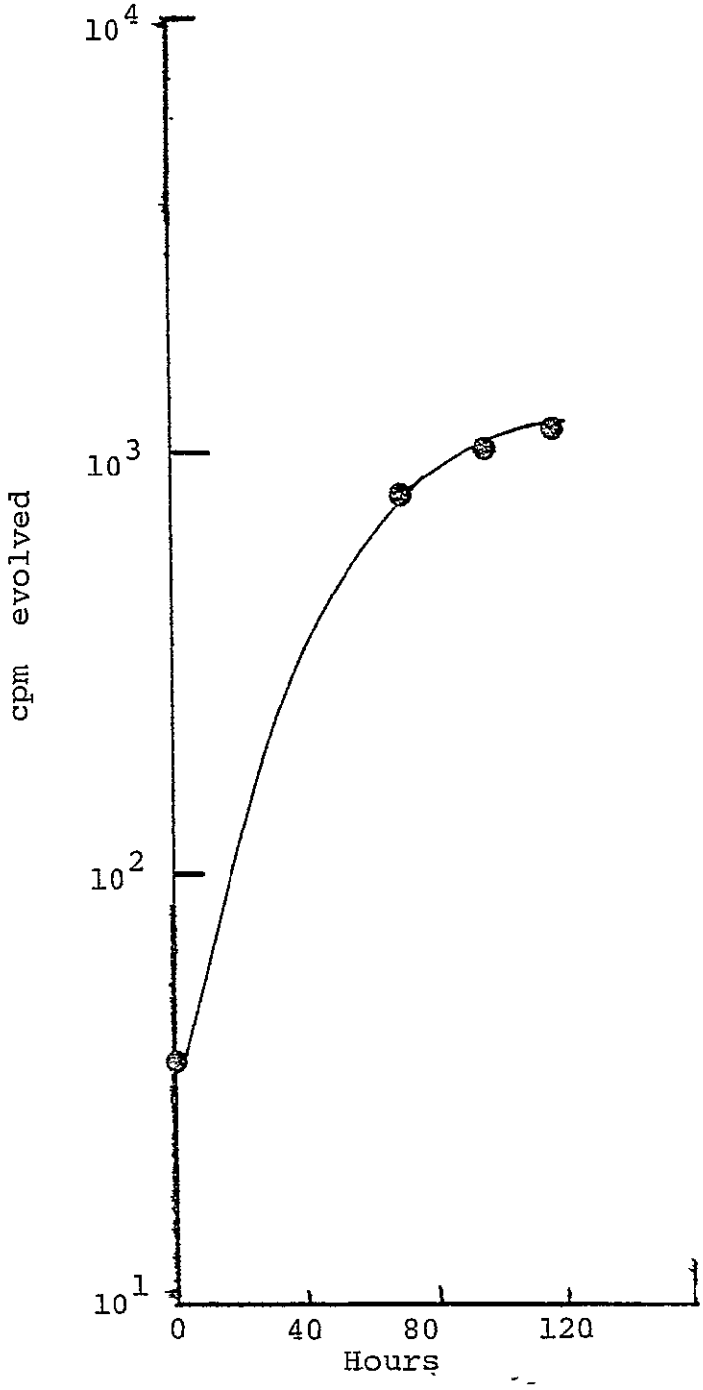
It should be noted that Medium K is identical to dilution Medium B with the addition of trace biotin. The 0.09 ml inoculum containing approximately 10^4 cells was added to each reaction vial containing 0.5 gm silica base and ^{14}C-labeled lactate (0.04 μCi) then added in a 0.01 ml volume. The system was sealed using a rubber septum and flushed with an anaerobic gas mixture (80% N_2, 10% H_2, 10% CO_2) sterilized by passage through a sterile 0.22 μ membrane filter. After five minutes of flushing at 800 ml/min, the

vials were counted in a liquid scintillation vial and transferred to a light box. Incubation conditions in the light box were at room temperature with an exposure of 125 footcandles of tungsten light. At various intervals, the vials were removed from the light box, placed in a liquid scintillation counter for monitoring $^{14}CO_2$ evolution, and then immediately returned to the appropriate incubation station. Typical metabolic responses obtained in the light box (Figure 12) indicate that under these conditions, $^{14}CO_2$ evolution plateaus within 72 hours.

In a further attempt to define conditions approximating those on Mars at which R. rubrum can metabolize lactate, a similar experiment has been conducted by placing the reaction vials in the Mars box and removing them as described above for periodic counting in the liquid scintillation counter. In addition to the anaerobic reaction conditions described above, the Mars box experiment also imposed a reduced temperature of 15°C and 2 - 4 hours per day of ultraviolet radiation, although these preliminary experiments were not conducted at reduced pressure. All reaction vials were also exposed to a light/dark regime of 16/8 tungsten light to accommodate photoheterotrophic metabolism. Because of the cyclic UV exposure over the two week experiment, these conditions imposed a more severe stress than those previously (Table 7) examined. R. rubrum metabolism in the Mars box was compared to that obtained by standard anaerobic procedures

Figure 12

Typical Metabolic Response of Rhodospirillum rubrum with ^{14}C-Lactate as Substrate



at 26°C in the light box. The results (Table 8) indicate that R. rubrum is marginally tolerant to the conditions defined by the Mars box experiment. Thus, of the duplicate reaction vials, one showed no metabolism while the other exhibited lactate metabolism at a significantly reduced rate. Future experiments will attempt to utilize these conditions or a somewhat less severe regime to optimize the R. rubrum response under conditions that still approach those of Mars but will permit responses from hardy organisms.

Table 8

Effect of Harsh Conditions on Rhodospirillum rubrum Metabolism

Vial Number	Atmosphere	Temperature (^{o}C)	Irradiation	Hours of Incubation	Location of Incubation	cpm Evolved
1	80% N_2,10% H_2,10% CO_2	26^{o}	Constant 125 ft candles tungsten	119	Light Box	1068
2	80% N_2,10% H_2,10% CO_2	26^{o}	Constant 125 ft candles tungsten	119	Light Box	1105
3	80% N_2,10% H_2,10% CO_2	15^{o}	Light/Dark tungsten (16/8 hours) 2-4 hrs. UV Exposure	336	Mars Box	0
4	80% N_2,10% H_2,10% CO_2	15^{o}	Light/Dark tungsten (16/8 hours) 2-4 hrs. UV Exposure	336	Mars Box	600

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4.0 STRESS CHALLENGES TO BASIC WARNING SYSTEM

In applying the Basic Warning System to test effects of a Mars sample, several modes of inhibition are possible. Viable organisms in the Mars sample could utilize the $^{14}CO_2$ end product of terrestrial metabolism, thereby producing an apparent inhibition. Such inhibition is typified by elementary ecosystem experiments reported earlier for combinations of algae and bacteria. A second mode of inhibition is one in which the Mars sample stresses the production of $^{14}CO_2$. It is imperative that the BWS be sensitive to these stresses. To delineate the BWS characterists in the presence of stress, a study has been conducted using Azotobacter chroococcum and Isolate Ps-3 as models for vital processes and Rhodospirillum rubrum as model for hardy organisms. Three types of stress have been considered. First is one in which a viable Mars sample inhibits terrestrial metabolism indirectly by competing for a substrate. The second is one in which a Mars organism directly by acting as a predator or by inhibiting a specific metabolic pathway at one or more reactive sites. The third type of stress considered is one in which the BWS is affected by the physical properties of the sample, such as elemental composition, rather than by the constituent Mars organisms. Representatives of each type of stress have been added to the model organisms to ensure the

required performance of the proposed scheme as a Basic Warning System.

4.1 Standard Procedures for Stress Studies

Experiments designed to test competition between two microorganisms for the same substrate combined Azotobacter and Ps-3 in one reaction vial. Standard cultures were grown as previously described (Section 3.1.1) and all inocula were prepared in dilution Medium B. Additional experiments in which a viable organism not expected to compete with Azotobacter or Ps-3 for the same substrate were also performed. These studies utilized Staphylococcus aureus which is unable to metabolize lactate.

Staphylococcus aureus cultures were grown by loop inoculation of 10 ml nutrient broth (Medium L) in a 125 mm screw cap tube. Cultures were incubated on a reciprocating shaker at room temperature (app. 26^{o}C) for 24 hours. An innoculum for an experiment was prepared by dilution of the culture in an appropriate medium depending on the organism with which it would be combined. In combination with Ps-3, dilution Medium B containing nitrate was used for both organisms. In combination with Azotobacter, dilution Medium M containing molybdenum and lacking nitrate was used for both organisms. Each Staphylococcus inoculum contained 10^3 cells in a 0.04 ml volume whereas inocula of Ps-3 or Azotobacter contained 10^3 cells in a 0.05 ml volume.

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Predator experiments utilized Uronema nigricans as a model. This organism, obtained by courtesy of Sharon Berk at the University of Maryland, is a unicellular heterotrophic ciliate that feeds on bacterial cells or cell debris. U. nigricans cultures were grown at room temperature in a 250 ml Erlenmeyer flask containing 125 ml Dilution Medium B or M depending upon whether cultures were designated for predator experiments with Ps-3 or Azotobacter, respectively. At monthly intervals, cultures were maintained by 1:1 dilution with fresh dilution medium and the daughter cultures incubated in 125 ml volumes. All cultures received weekly feedings with 10 ml of a Ps-3 culture (app. 10^8 cells/ml) sterilized in advance by autoclaving. Cultures were enumerated by adding 0.01 ml Lugol's iodine stain to 0.05 ml culture aliquots:

Lugol's Iodine Stain

Iodine	5 g
Potassium Iodine	10 g
distilled water	100 ml
Adjust pH to approximate that of material to be stained	

Predator stress experiments with Uronema were monitored by the air sampling technique described for monitoring photosynthesis (see Section 3.1.2). This technique was necessary to accommodate Uronema which would not grow on the soil base or in the presence of the fluor strips required for the Buddemeyer double vial technique used for all other studies (see below). To inoculate predator experiments, 0.1 ml aliquots containing approximately 50 starved (one week) Uronema cells were added directly to the reaction vial containing 1.85 ml

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growth medium. Approximately 10^3 cells of Ps-3 or Azotobacter, as appropriate, were also added in a 0.05 ml volume followed by 0.01 ml of ^{14}C-labeled lactate containing 0.32 µCi. The vials were sealed with rubber septums and monitored for $^{14}CO_2$ evolution. At appropriate intervals, further 0.01 ml additions of labeled lactate were made and aliquots were removed from the reaction vials for monitoring $^{14}CO_2$ levels and for enumeration. Predator populations were enumerated as described as above whereas bacterial populations were enumerated by plate counting dilutions of a 0.05 ml aliquot.

All stress experiments other than those with Uronema utilized the repeated addition technique and the Buddemeyer system to monitor $^{14}CO_2$ evolution as previously described (Sections 3.1.1 and 3.2.3). Experiments with Ps-3 and Azotobacter were aerobic whereas those with Rhodospirillum rubrum were anaerobic in the light. The inoculum for each test organism contained 10^3 bacteria in a 0.05 ml volume. This inoculum was prepared as previously described and added onto a 0.5 g silica or soil base, as appropriate. Within this base was incorporated the competitive organisms, specific metabolic inhibitors, or physical parameters under consideration as stress agents. The reaction was started by the addition of 0.01 ml ^{14}C-labeled lactate containing 0.04 µCi; further lactate additions were made at approximately one week intervals.

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Specific metabolic inhibitors tested as stress agents were 5-phenyl-1,10-phenanthroline (Sigma), sodium azide (Fisher Scientific), diphenylamine (Fisher Scientific), iodoacetate (Eastman Chemical), and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU, DuPont). Solutions of phenanthroline, diphenylamine, and DCMU were prepared in acetone at an appropriate concentration according to the amount desired in the stress experiment. These inhibitors were incorporated directly into the silica base by adding the acetone solution to the base just prior to autoclaving. Volumes of 0.01 ml were used for phenanthroline and diphenylamine solutions whereas a volume of 0.1 ml was used for DCMU solutions. Vial caps were loosened during autoclaving to ensure acetone evaporation. This incorporation method ensured sterility and a mixing of the stress agent into the silica base in a uniform manner. On the other hand, because sodium azide and iodoacetate are not stable to autoclaving, these agents had to be prepared in an appropriate medium and added to the silica base after autoclaving. Solutions of these agents were prepared in Dilution Medium B for experiments with Ps-3 and in Dilution Medium M lacking nitrate but containing trace molybdenum for experiments with Azotobacter. Solutions were delivered in a 0.01 ml volume at a concentration calculated to provide the desired molarity in the stress experiment. Final molarity of the reaction mixture was calculated assuming a 0.5 g silica volume plus a 0.1 ml liquid volume from all additions for a total volume of 0.6 ml.

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For experiments in which physical stress was to be provided, soil was substituted for or added to silica base such that a total 0.5 g base existed in all experiments. Stress soil constituents were selected to simulate physical or chemical properties delineated by Viking data from analyses of Mars surface samples. To each defined base was added 0.05 ml containing 10^3 cells from either Ps-3, Azotobacter, or Rhodospirillum cultures. ^{14}C-labeled lactate (0.04 μCi) was added in a 0.01 ml volume at intervals appropriate for repeated addition experiments. At the termination of each experiment, vials were enumerated by standard techniques (Section 3.1.1) to determine cell numbers.

4.2 Effects of Specific Stresses

4.2.1 Substrate Competition

Addition of a Mars sample to the Basic Warning System could impose a stress related to competition between the BWS organism and a Martian organism for a common substrate. Experiments designed to characterize this type of stress utilized two organisms within the same Buddemeyer reaction vial. Results with Ps-3 and Azotobacter are presented in Table 9. As shown, both organisms independently respond to three ^{14}C-labeled lactate additions separated by 168 hour intervals. However, the magnitude of the responses from the two species are sufficiently different to be characteristic. When both organisms are mixed in a competition

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Table 9

Substrate Competition
Between Ps-3 and *Azotobacter*

Addition Number	cpm Evolved Per Addition		
	Azotobacter	Ps-3	*Azotobacter* and Ps-3
1	3592	7657	3256
2	6393	5172	5273
3	1764	4350	1773

Additions of 0.05 ml ^{14}C-labeled lactate were made at 168 hour intervals to each Buddemeyer Reaction Vial containing *Azotobacter* or Ps-3 cells singly or combined, as indicated. For each injection, counts evolved are given as the increase over the preceeding plateau.

experiment, the resultant response to lactate is neither additive nor is it an average of the independent responses. Rather, it appears characteristic of the Azotobacter response. This result could be interpreted either that mixed metabolism results in altered metabolic rates from both organisms or that Azotobacter partially inhibited Ps-3 metabolism, or that Azotobacter successfully competed for all lactate, thereby preventing any metabolism by Ps-3. The latter interpretation resembles one in which no substrate competition occurred. Thus, Table 10 presents results of experiments in which Staphylococcus was mixed with either Azotobacter or Ps-3. As shown, Staphylococcus does not utilize lactate nor does it interfere with the metabolism of either of the other two organisms.

These results indicate that a stress whereby a viable Mars organism is present may or may not be detected by the BWS. The sensitivity of the BWS can be improved by utilizing several different terrestrial organisms and substrates to monitor possible effects. However, the results emphasize the need to consider negative results in the BWS with caution.

4.2.2 Predation

Mars organisms could also inhibit metabolism by predation upon the indicator organisms of the Basic Warning System. Predation stress experiments have combined the ciliate Uronema with Ps-3 or Azotobacter by methods developed

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Table 10

Effect of Staphylococcus on Substrate Utilization by Ps-3 or Azotobacter

Addition Number	cpm Evolved Per Addition				
	Staphylococcus	Ps-3	Azotobacter	Ps-3 plus Staphylcoccus	Azotobacter plus Staphylcoccus
1	- 65	4797	2957	5174	3111
2	+109	+660	3681	+570	4233
3	+ 20	4355	-196	2796	-193

Additions of 0.05 ml ^{14}C-labeled lactate were made at 168 hour intervals to each Buddemeyer Reaction Vial containing Staphylococcus, Ps-3, or Azotobacter singly or combinations of Staphylococcus with Ps-3 or Azotobacter, as indicated. For each injection, counts evolved are given as the increase over the preceeding plateau.

and reported in the previous section.

The results of experiments with Azotobacter and Uronema are presented in Figure 13. As shown, the response of Azotobacter alone to an initial ^{14}C-labeled lactate addition shows a lag typical of cultures grown on nitrate-free media that probably represents adaptation to heterotrophic metabolism. A plateau in gas evolution is reached after approximately 70 hours. During this time, Azotobacter cell numbers continually increase. Addition of Uronema to the Azotobacter culture changes the character of the kinetic response by eliminating the lag phase. Although the reason for this is unknown, it cannot be related to addition of extraneous bacterial cells present in the Uronema culture because bacterial enumerations indicate a similar initial cell number in both reaction vials with and without the presence of Uronema. The elimination of the lag also does not appear related to Uronema cells which show an immediate decrease in number. This decrease may be related to the low initial number of bacterial cells (10^3) since it is known that optimal Uronoma feeding requires a higher bacterial concentration and that Uronoma starves and dies at low bacterial concentrations. However, as the total bacteria cell population increases with lactate metabolism, a Uronema bloom occurs, after which the rate of increase in Azotobacter cell numbers decrease, presumably due to Uronema grazing. The lower Azotobacter numbers

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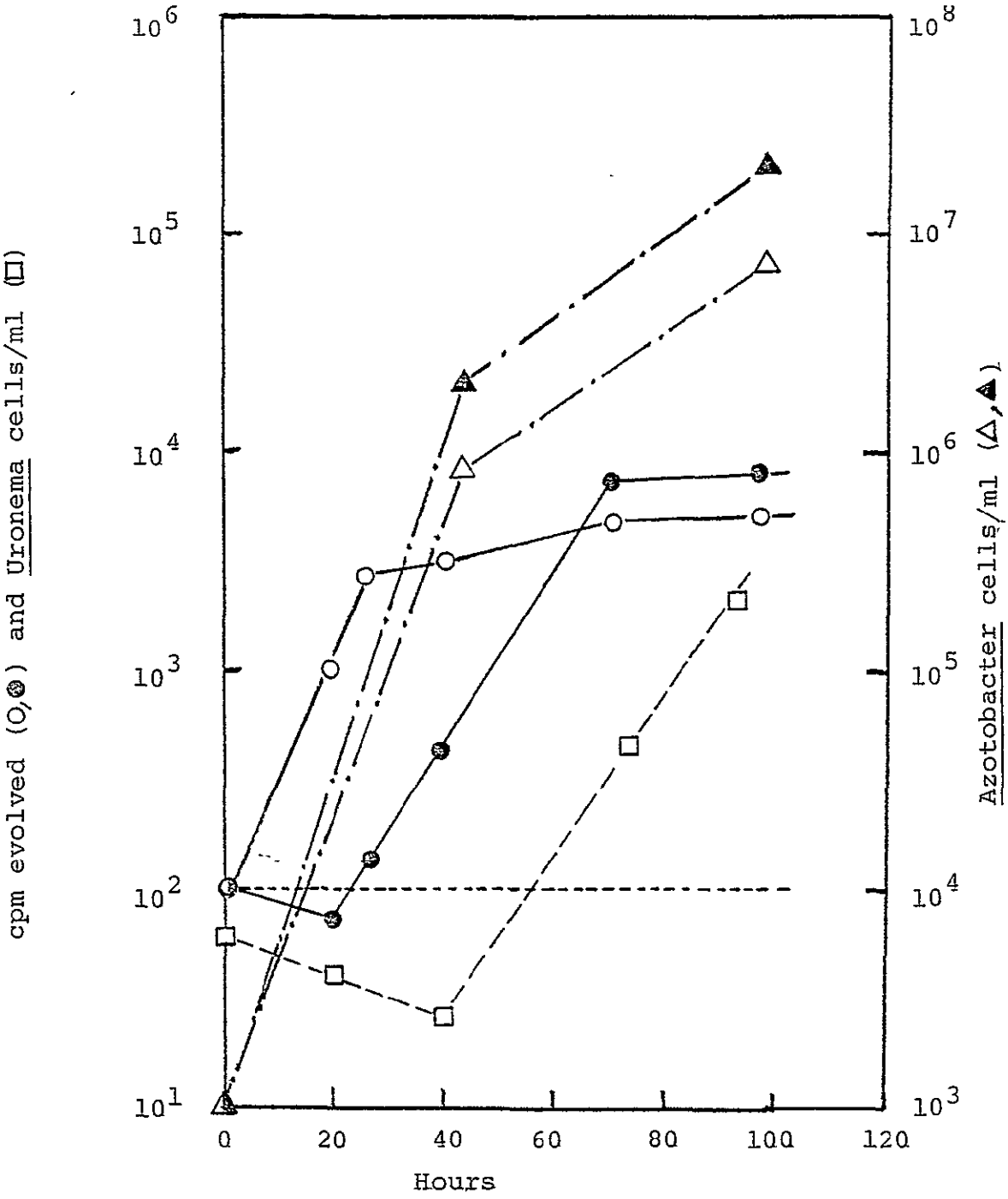
Figure 13

Effect of Uronema on Azotobacter Metabolism



^{14}C-lactate metabolism by Azotobacter was studied in the presence (O--O) and absence (●--●) of the predator Uronema. Radioactivity from sterile medium alone is also shown (----). Azotobacter cells/ml were also determined in the presence (△-·-△) and absence of Uronema (▲-·-▲). Predator cells/ml (□--□) were also determined at various intervals.

in the presence of Uronema may account for the reduced plateau in gas evolution.

Preliminary experiments have also been conducted with Azotobacter in the presence and absence of Uronema using the repeated addition technique. The results show a reduced second and third response in the presence of Uronema, possibly because fewer bacterial cells are present due to Uronema grazing or because Uronema may influence the availability of lactate. However, the results are also complicated by the apparent ecosystem effect whereby cyclic population changes may be occurring with both species. This has presented a problem in data interpretation and further work is necessary to fully delineate the predator effects. Eventually it may be possible to develop a cyclic kinetic response capable of recognizing the presence of a predator in the Basic Warning System.

Preliminary predation experiments have also been conducted between Uronema and Ps-3. The results presented in Figure 14 show that the effect of the predator on Ps-3 lactate metabolism is considerably more difficult to detect or may not be occurring. Thus, metabolic responses to repeated additions for Ps-3 are somewhat enhanced by the presence of Uronema although the shape of each kinetic response remains unchanged by the presence of Uronema.

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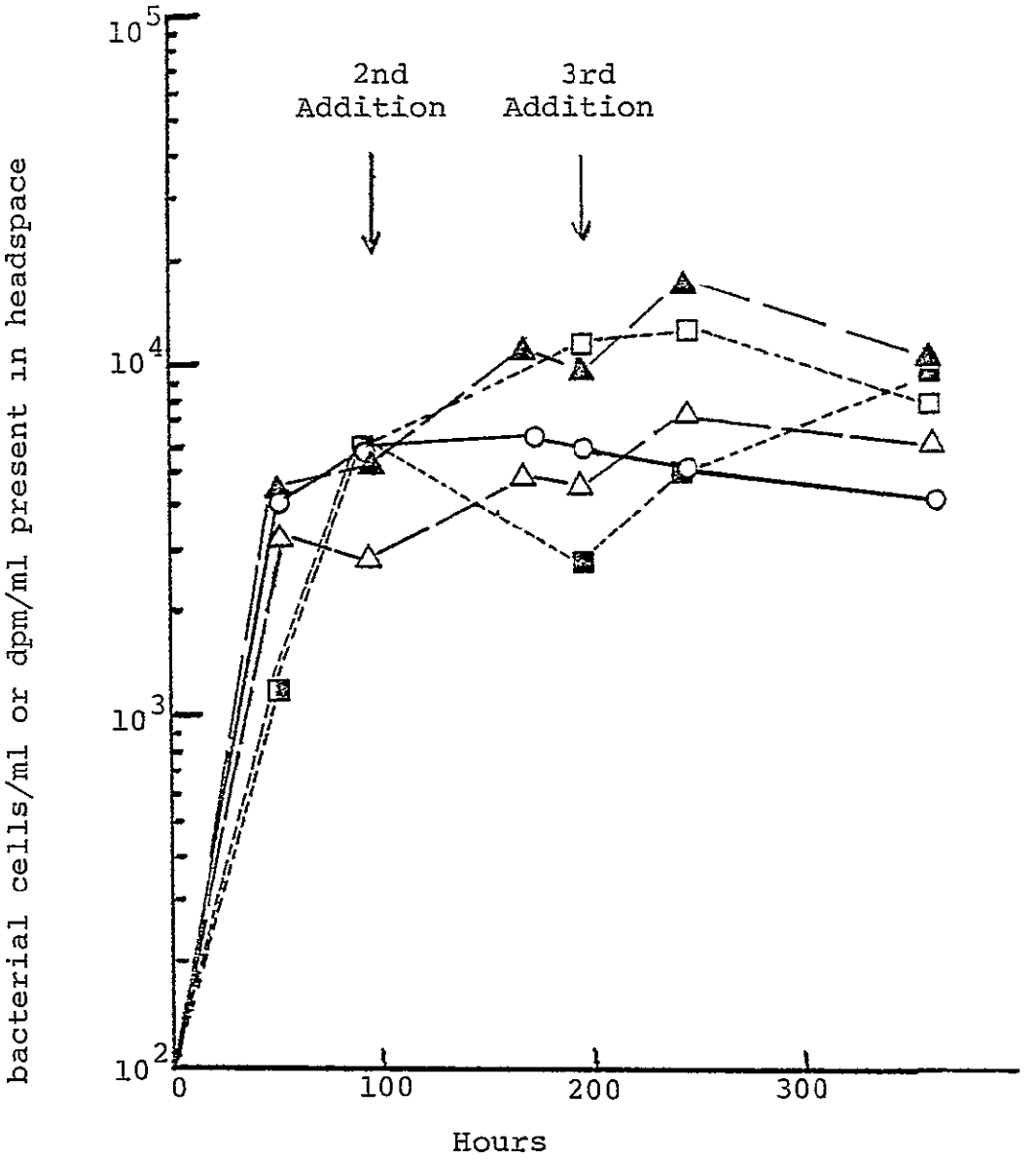
Figure 14

Effect of Uronema on Ps-3 Metabolism



^{14}C-lactate metabolism by Ps-3 was studied by the repeated addition technique in the presence (▲--▲) and absence (△--△) of the predator Uronema. The second and third substance addition occurred as indicated. The effect of Uronema on Ps-3 metabolism for only one initial substrate addition (0——0) was also examined. Bacterial cells/ml were enumerated in the presence of Uronema for both the single addition (□---□) and the repeated addition (▣---▣) reaction. Essentially no radioactivity was evolved from medium alone.

Cyclic population numbers in Uronema are also evident during these repeated additions, possibly related to previously described (Figure 12) fluctuations in bacterial populations during the repeated addition technique. Fluctuations in Uronema populations are not as evident in combination experiments with Ps-3 which receive only one lactate addition. This may reflect the constancy of bacterial populations over the duration of single addition experiments (see "Kinetics of Prolonged Response").

Predation, then, may or may not be detected by the proposed ^{14}C metabolism bases Basic Warning System, depending on the indicator organism. A more reliable indicator of predation would be cell enumeration of the indicator, although even here complications arise due to the cyclic populations previously observed during the repeated addition technique. Nonetheless, since in some cases predation can be detected by this technique, it would be of interest to pursue studies with Azotobacter to establish a "library signature" for predation. Again, however, negative results with a Mars sample would not necessarily imply the absence of a predator.

4.2.3 Specific Metabolic Inhibitors

A viable surface sample obtained from Mars could also inhibit specific terrestrial pathways, possibly through the production of toxic metabolic end products. In

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order to characterize this type of stress on our proposed Basic Warning System, four metabolic inhibitors have been examined for effects on Ps-3 and on Azotobacter metabolism of ^{14}C-labeled lactate metabolism and one for effects on Rhodospirillum metabolism. The metabolic inhibitors used in this study are iodoacetate, sodium azide, phenyl-phenanthroline, diphenylamine and DCMU, each prepared and added to the reaction mixtures as described in "Standard Procedures for Stress Studies". Iodoacetate is a well-known inhibitor of carbohydrate metabolism, historically used to inhibit triosephosphate dehydrogenase and enolase (10) although it also has a more general action as a sulfhydryl agent (11). Sodium azide is a respiratory poison acting classically at the site of cytochrome oxidase (11) whereas both azide and phenanthroline have been used as inhibitors of nitrogen fixation (12). Phenanthroline and sodium azide are also known chelators (11,13) used extensively to detect enzymatic reactions with metal prosthetic groups. Diphenylamine has been reported to inhibit caroteniod synthesis in photosynthetically growing cultures of Rhodospirillum rubrum (10) and is also known to bind to purine deoxyribosides (14). DCMU (also known as Diuron) is an herbicide known to block non-cyclic photophosphorylation in organisms such as R. rubrum (15).

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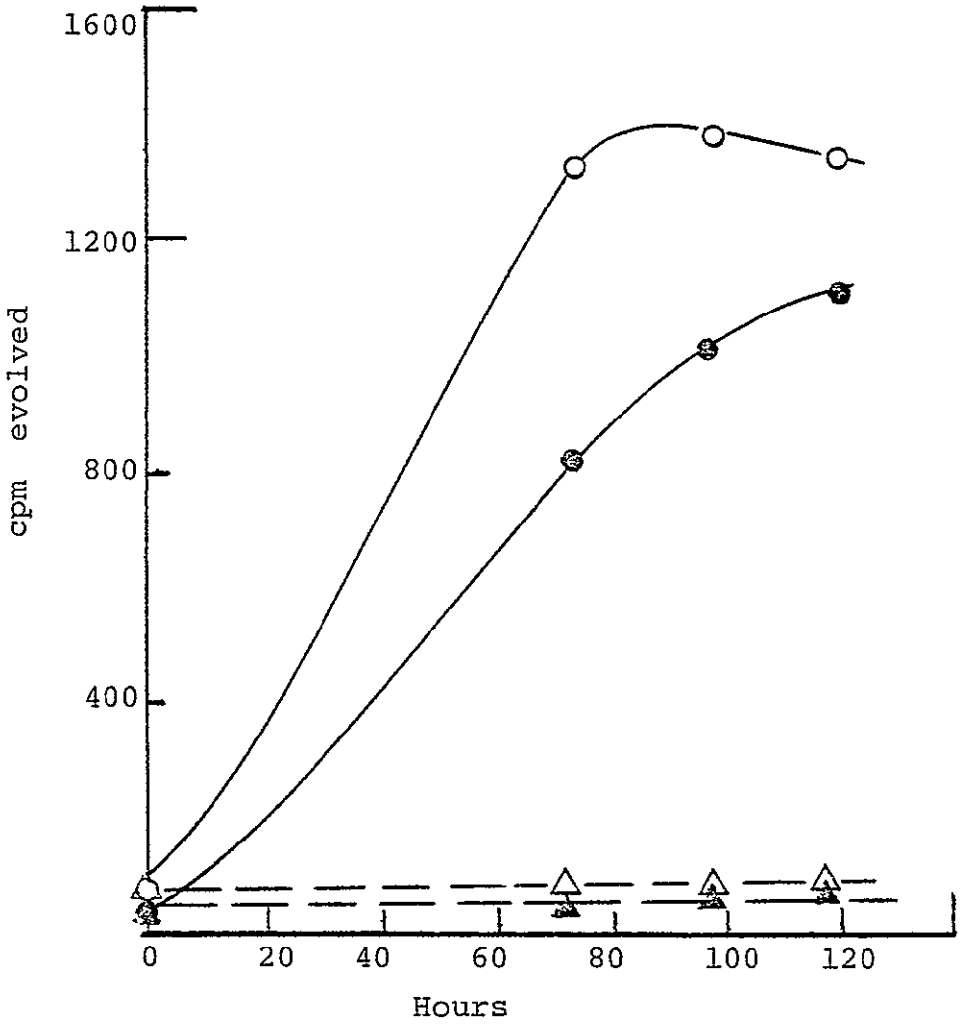
Previous results (1) with Rhodosprillum rubrum showed that 10^{-2}M diphenylamine and 10^{-3}M cyanide produced a 74% and 85% inhibition, respectively, of ^{14}C-lactate metabolism. On the other hand, recent experiments (Figure 15) with 10^{-3}M DCMU and R. rubrum failed to produce an inhibition and may in fact have stimulated lactate metabolism. This failure to inhibit may result from insufficient inhibitor concentration, a permeability barrier, or because the mechanism of DCMU action on non-cyclic phosphorylation does not affect lactate metabolism under the conditions of the experiment. Nonetheless, the results indicate that the BWS may not necessarily detect an inhibitor of terrestrial metabolism and point out the desirability of utilizing several indicator organisms.

Results obtained with four of these inhibitors combined with either Ps-3 or Azotobacter are presented in Table 11. High inhibitor concentrations were used in an attempt to overcome possible permeability barriers of the indicator organisms and to ensure an inhibited metabolic response. The results show that both organisms are indeed sensitive to these inhibitors. However, little difference in percent inhibition was observed between Ps-3 and Azotobacter for each inhibitor despite the known differences in bacterial metabolism and in mechanism of inhibitor action. For example, Azotobacter was expected to be considerably more sensitive than Ps-3 to agents inhibiting nitrogen metabolism.

Figure 15

Effect of DCMU on Rhodospirillum rubrum Metabolism



^{14}C-lactate metabolism by R. rubrum was studied in the presence (O—O) and absence (●—●) of 10^{-3} M DCMU. Sterile medium with (△--△) and without (▲--▲) DCMU was also examined.

C-2

Table 11

^{14}C-lactate Metabolism by Ps-3 and Azotobacter

Inhibitor	Concentration	Average Percent Inhibition of Plateau Level	
		Ps-3	Azotobacter
Iodoacetate	10^{-2} M	100	99
Sodium Azide	10^{-2} M	99	100
Phenylphenanthroline	10^{-2} M	85(55,100,100)	53(0,60,100)
	$2x10^{-3}$ M	19(12,10,33)	100
Diphenylamine	10^{-2} M	99	39(5,12,100)
	$2x10^{-3}$ M	12(0,12,11)	0

Reactions were conducted in triplicate by the Buddemeyer technique. Where results vary markedly, the constituent percent inhibitions are given in parentheses after the average value.

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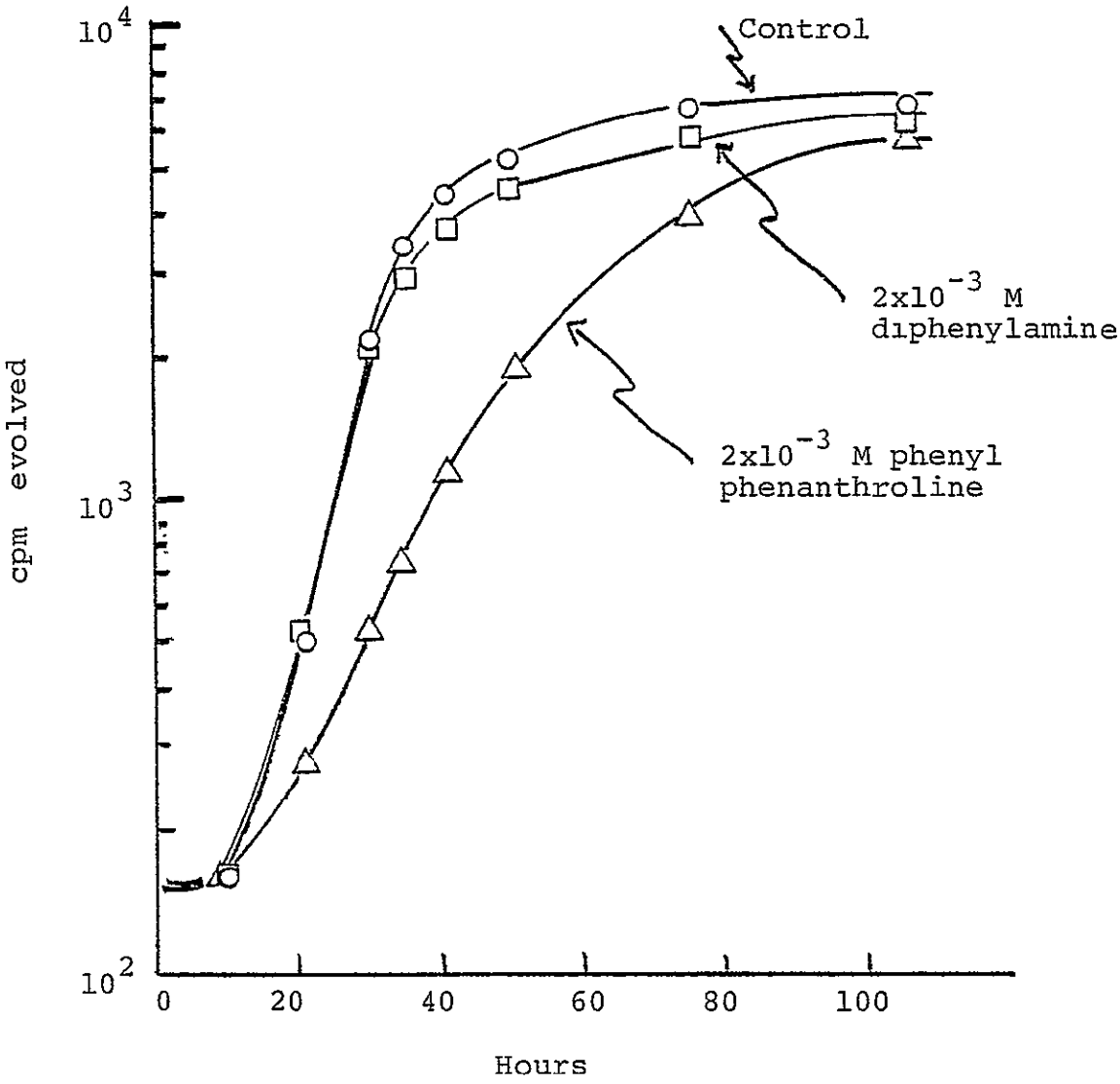
Perhaps the high inhibitor concentrations used in this study resulted in more general inhibitory action rather than site specific action. This is typical for inhibitors such as azide which at low concentrations affects cytochrome oxidase but at high concentrations affects many other metal requiring enzymes.

Attempts to use lower inhibitor concentrations of diphenylamine or phenanthroline to demonstrate more specific inhibition of selected pathways were somewhat more successful. Thus, at lower inhibitor concentrations, Azotobacter was more sensitive to phenanthroline whereas Ps-3 was somewhat more sensitive to diphenylamine. An examination of the kinetics of these responses is presented in Figure 16. As shown, each agent produces a small inhibition of the plateau level finally achieved. However, examination of the kinetics of $^{14}CO_2$ evolution in the presence of phenanthroline indicates an even larger inhibition of initial rates that would not have been obvious had only plateau levels been examined. This capability of the proposed BWS in continuous monitoring thus provides a powerful tool for detection of metabolic inhibition.

It should be noted that considerable variability in percent inhibition was observed with phenanthroline and diphenylamine. Both of these agents were incorporated into the silica base as described in "Standard Procedures for Stress Studies" by mixing an acetone solution of the inhibitor with

Figure 16

Effect of Inhibitors on Ps-3 Metabolism



the base and then evaporating away the acetone. Although this technique should provide an excellent model of an inhibitor present in a Martian sample, it apparently requires further development to obtain reliable data. Despite these difficulties, the overall results of this study show that the proposed BWS is generally effective in detection of metabolic inhibitors. Further refinement is required, however, to fully demonstrate its capabilities in detecting differential inhibition of major terrestrial pathways. On the other hand, since data with R. rubrum show that a known inhibitor is not necessarily detected by the system, the results again emphasize the importance of considering negative results with caution.

4.2.4 Physical Stresses

Another type of inhibition that could affect the metabolism of the Basic Warning System organisms is that resulting from physical properties of the Mars sample. These could be specific constituent compounds or elevated concentrations of specific elements. Using methods described under "Standard Procedures for Stress Studies", both Ps-3 and Azotobacter have been used to examine the ability of the BWS to detect stresses in this category. Basically, stress agents were mixed with or substituted for the 0.5 g silica base and all experiments conducted aerobically in a Buddemeyer double vial system.

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The stress agents selected were designed to test the effects of known or postulated properties of the Mars sample based on results from the Viking '76 Mission. Table 12 presents the composition and elemental analysis of a Mars analog prepared by the Inorganic Analysis Team to match as closely as possible the elemental analysis obtained on a Mars sample from Chryse (Mars Landing Site #1). (The manner of preparation of this Mars analog assured its sterility). To obtain an approximation of the possible inhibition of a Mars sample, an experiment was conducted whereby Ps-3 was placed on a 0.5 g base containing various mixture ratios of silica and the Mars analog. The results (Table 13 and Figure 17) show that the Mars analog is indeed inhibitory to Ps-3 metabolism. When the analog was substituted for the silica base, inhibition was complete. Even when present at a 1:50 dilution, 12% inhibition was evident.

An attempt was made to delineate the inhibitory agents in the Mars analog by studying stresses imposed by specific elements present. In this experiment, both Ps-3 and Azotobacter were subjected to a mixture containing 12.7% iron (as Fe_3O_4), 6.2% magnesium, and 5.0% sulfur (both as $MgSO_4$). The addition of these salts lowered the silica content of the base from 100% to 40%, about double the estimated 19% on the surface of Mars. Because it has been

Table 12

Composition of Mars Analog Soil

Constituents of Mars Analog Soil	Elemental Composition of Mars Analog Soil	
51.7% Montronite (RN)	Element	Percent
25.5% Montmorillonite (WYO)		
9.4% Kieserite	Na	.35
6.0% Calcite	Mq	2.65
5.0% Hematite	Al	3.84
3.0% Magnetite	Si	19.15
100 % By Weight	P	.01
	S	2.26
The mixture was prepared by and obtained from A. Baird of the Viking Inorganic Analysis Team	Cl	.89
	Ar	.08
	K	.15
	Ca	5.17
	Ti	.18
	Fe	15.71
	C	.78
	O	48.78
	Total	100.00

Table 13

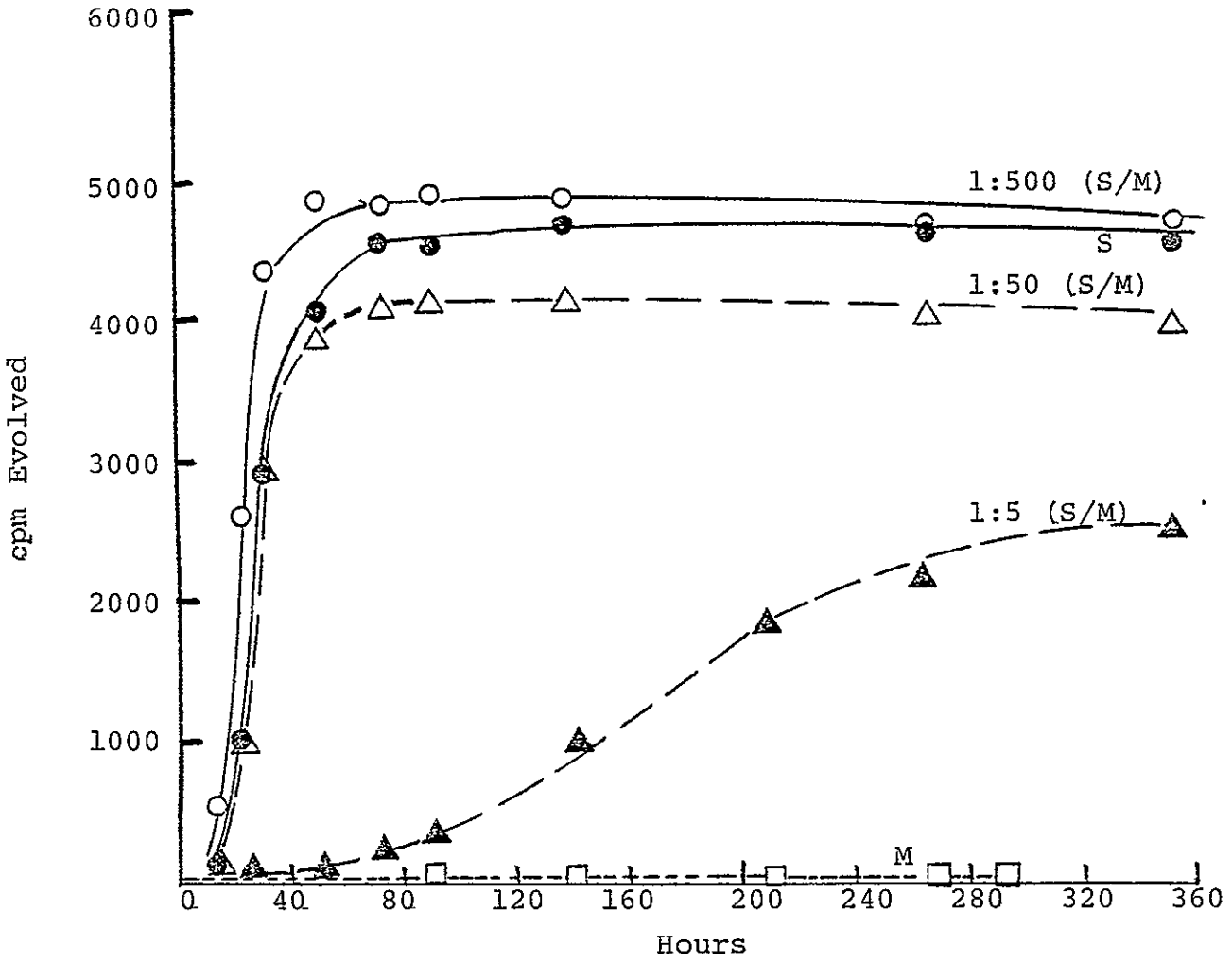
Effect of Mars Analog Soil on Ps-3 Metabolism of ^{14}C-Lactate

Grams Present in Reaction Base		Dilution of Mars Analog	Average cpm Evolved	Average Percent Inhibition by Mars Analog
Mars Analog	Silica			
0.500	0	0	0	100
0.100	0.400	1:5	2712	41.5
0.010	0.490	1:50	4041	12.5
0.001	0.499	1:500	4766	0
0	0.500	∞	4639	--

The silica base used in the Buddemeyer technique was diluted with varying amounts of Mars Analog soil maintaining the total volume of the base at 0.5 g.

Figure 17

Effect of Mars Analog Soil on
Metabolic Response of Ps-3



^{14}C-lactate metabolism by Ps-3 was monitored by the Buddemeyer technique. The silica base was diluted with varying amounts of Mars Analog Soil such that the total volume of the base remained constant at 0.5 g. Dilutions are indicated as parts silica to parts Mars Analog. Results using only silica base (S) or the Mars Analog alone (M) as base are also shown.

proposed that the Mars surface is basic (16), an additional stress study was conducted at pH 8.5, with all solutions and the silica base adjusted to pH 8.5 using sodium hydroxide. Another stress vial imposed 0.16% calcium added as calcium peroxide since peroxides have been proposed (3) to account for the positive results obtained on Mars by the Labeled Release experiment. Cells from each reaction vial were enumerated at the termination of the 290 hour experiment.

The metabolic results obtained with the elemental stress agents are shown in Tables 14 and 15 for Ps-3 and Azotobacter, respectively. As shown, stresses imposed either by elevated calcium content or by a mixture of elevated iron, magnesium, and sulfur were inhibitory to the metabolism of both organisms. Where tested, a second substrate addition performed after 100 hours did not overcome this inhibition. An elevated pH, on the other hand, was more inhibitory to Azotobacter than to Ps-3. Cell enumerations at the end of the experiments showed viable cells only in the controls without stress and in elevated pH experiments with Ps-3 which was not inhibited by a first substrate addition and only partially inhibited by second and third substrate additions. No inhibition was seen, however, in the number of viable cells present.

Table 14

Exposure of Ps-3 to Various Physical Stresses

Stress Added	First Substrate Addition		Second Substrate Addition		Third Substrate Addition	
	Average cpm Evolved	Percent Inhibition	Average cpm Evolved	Percent Inhibition	Average cpm Evolved	Percent Inhibition
None	5828	0	6998	0	8301	0
12.7% iron + 6.2% magnesium + 5.0% sulfur	0	100	0	100	--	--
0.16% calcium as calcium peroxide	0	100	--	--	--	--
pH 8.5	6871	0	2291	67	7526	10

Additions of ^{14}C-labeled lactate were made at 0, 96 and at 189 hours. Radioactivity evolved for each addition is presented as the increase over the plateau from the previous addition.

Table 15

Exposure of Azotobacter to Various Physical Stresses

Stress Added	First Substrate Addition		Second Substrate Addition	
	Average cpm Evolved	Percent Inhibition	Average cpm Evolved	Percent Inhibition
None	3634	0	6437	0
12.7% iron + 6.2% magnesium + 5.0% sulfur	0	100	0	100
0.16% calcium as calcium peroxide	0	100	0	100
pH 8.5	36	99	0	100

The second addition of ^{14}C-labeled lactate was made at 100 hours. Radioactivity evolved for each addition is presented as the increase over the plateau from the previous addition.

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An additional physical stress considered for Vital Process BWS organisms was that imposed on the organisms by testing in a cold environment. An experiment with Ps-3 compares metabolism conducted at room temperature (app. 26°C) to that obtained at 10°C. The results (Figure 18) show that the lower temperature inhibits the kinetic rate of $^{14}CO_2$ evolution but not the magnitude of the plateau obtained following either a first or second substrate addition.

An experiment was also conducted in which Rhodospirillum rubrum was contained in the harsh Mars-like conditions defined in Section 3.2.3 (i.e., 15°C, anaerobic, UV radiation) and subjected similarly to elevated concentrations of iron, magnesium, sulfate, and calcium and to elevated pH (8.5). However, although metabolism was observed in the Mars Box controls, albiet at a significantly reduced rate, the additional stress agents resulted in total inhibition of metabolism. It appears that future efforts must define less harsh Mars-like conditions for R. rubrum in order to utilize this organism for such stress studies.

Studies with physical stress agents thus indicate that inhibition observed in the proposed BWS must also be regarded with caution since it does not necessarily indicate viable Mars organisms. However, a continued study of

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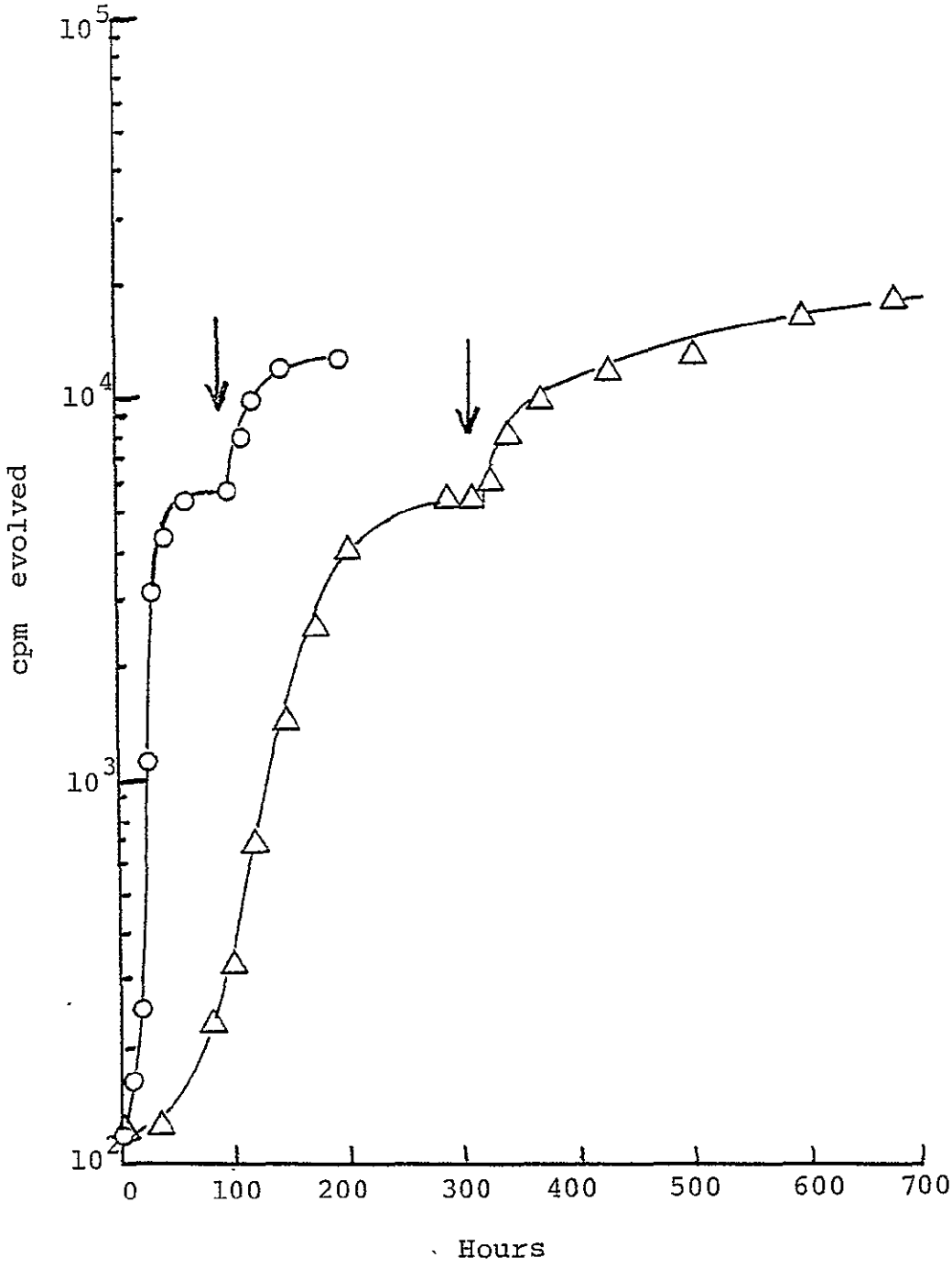
Figure 18

Cold Stress of Ps-3 Metabolism



Ps-3 metabolism of ^{14}C-lactate at 26°C (O—O) and at 10°C (Δ—Δ). For each reaction vial, second substrate additions were performed at the indicated time.

this nature using varying concentrations of the constituent Mars elements should permit development of a library of characteristic responses. By using varying amounts of the Mars sample in the BWS, then, this may provide a mechanism for determining whether inhibition results from a physical or biological agent.

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5.0 CULTURE PRESERVATION AND RE-VITALIZATION

A major consideration for the Basic Warning System is the transport and maintenance of viable indicator organisms for use in examining a Mars sample on Mars or in orbit around Mars and in transit to Earth. The flight procedure for Viking '76 required biological constituents to be in storage approximately one year prior to launch. Transit time to Mars required an additional 11 months storage prior to the initial use of these constituents. Similar storage periods are anticipated for the proposed BWS along with additional storage periods between tests at Mars and in transit back to Earth. Such requirements could impose a system lifetime extending three or more years. Although problems related to the storage and transport of radioisotopes were solved for the Viking Labeled Release experiment, problems related to long-term storage of cultures during interplanetary flight have not previously been considered.

In order to accommodate the culture storage requirements, several methods have been examined for long term maintenance with revitalization of organisms upon command. Two approaches have been considered, one in which cultures are maintained in a state of "suspended animation" and the other in which growing cultures are maintained by providing necessary nutrients at appropriate intervals. The methods considered for each type of approach are considered below:

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5.1 Preservation in a Stable State

The method preferred for long-term cell culture preservation is one in which a stable state can be maintained, thereby eliminating the necessity for culture transfer and general maintenance. Such a stable culture would also have the advantage of genotypic and phenotypic integrity, thus eliminating the possibility of culture alteration or mutation over the duration of the flight experiment.

Common techniques for maintaining a stable culture are lyophilization, low temperature freezing, and the use of resistant cell forms such as bacterial spores and algal akenites. Low temperature freezing in liquid nitrogen followed by heat revitalization exhibits a high and reproducible rate of cell recovery. However, the requirement for a sufficient amount of liquid nitrogen to maintain a continued low temperature throughout the duration of a mission may be prohibitive. Resistant cell forms are also probably not suited to use in the BWS by virtue of the fact that such forms occur in the presence of external stress. Thus, after revitalization of a culture, it may not be possible to distinguish whether external stresses harm metabolic processes or cause formation of a resistant form.

Of the techniques available for maintaining a stable population, lyophilization, or freeze-drying, is probably most applicable. This technique is long-established for

maintaining a stable state known as "cryptobiosis" whereby bacteria and some higher organisms, such as rotifers, can be preserved as long as 40 years until revived by the addition of water or other appropriate medium (17). All lyophilization manipulations could be performed prior to launch and cultures maintained at ambient temperatures throughout the mission, provided these temperatures remained below some limit such as 35°C.

In applying lyophilization to the BWS, one can envision the lyophilized inoculum distributed into a series of sealed experimental chambers available for any given experiment as required. Upon breaking a desired seal, cells could be revitalized by suspension in an appropriate medium. Experiments could then be conducted directly in the lyophilization vial or the inoculum could be transferred to a separate experimental chamber. Standarization of the lyophilization procedure would ensure a uniform inoculum throughout all experiments performed regardless of the duration of the BWS.

Lyophilized cultures are typically frozen and resuspended in a rich medium consisting of a high concentration of a carbon source and all essential growth requirements (18). According to the National Collection of Type Cultures and the National Collection of Industrial Bacteria, reductions of one to two logs are typically obtained following lyophilization in rich media (18). However, such nutrients could

interfere with the experiments in consideration for the proposed BWS. In order to determine the applicability of the lyophilization technique to the BWS, a study was undertaken in which lyophilized cells were frozen and resuspended in a minimal medium. Although complete recovery was not anticipated, the experiments attempted to define conditions for attaining uniform recovery rates with a sufficient population to serve as inoculum.

Lyophilization studies were conducted with cultures of the Ps-3 Isolate, Pseudomonas flourescens, Azotobacter chroococcum, and Rhodospirillum rubrum grown by standard methods. In preparation for the lyophilization studies, a loop from an active culture was used to inoculate each of four 125 mm screw cap tubes containing 10 ml appropriate growth medium. Preliminary growth curve studies indicated that each species was in logarithmic growth after 24 hours shaking at 26°C. The four culture tubes were then combined and the 40 ml volume distributed into two sterile 50 ml polyethylene centrifuge tubes. The tubes were capped and centrifuged in a RC2B Sorvall centrifuge at 6800 x g for 10 minutes at 4°C. The supernatants were discarded and each of the two pellets resuspended in 5 ml of a wash solution consisting of 0.1% lactate. The combined 10 ml was centrifuged a third time. The washed pellet was finally suspended in 5 ml of the 0.1% lactate solution and 0.5 ml

aliquots transferred to sterile 2 ml prescored lyophilization ampoules (D23 Kimball). An additional 0.5 ml aliquot was transferred to 4.5 ml appropriate dilution medium for inoculum titer. Lyophilization ampoules were shelf frozen in a dry ice acetone bath and then attached by valve adapters to a LABCONCO bench mount freeze dryer. They were dried for 5 hours under vacuum (less than 0.15 torr as monitored by a McLeod Gauge #75850) using a 1/3 horsepower GCA/Precision Scientific vacuum pump (Model #5XBH002D). Care was taken to maintain consistent cooling and evacuation procedures since these variables are known to affect survival rates (18). The vials were then sealed with a gas-oxygen torch while still attached to the drying apparatus under vacuum. To prevent rapid cooling which frequently resulted in ampoule cracking, the vial seals were next placed in the flame of a Bunsen burner for 2 to 3 minutes before cooling. After cooling, seals were inspected for possible breaks. Where such breaks or cracks were evident, the ampoules were discarded since preliminary experiments show few or no survivors from cracked ampoules. Lyophilized vials were stored at room temperature until vial seals were broken at the prescored neck and the contents resuspended in 1 ml of an appropriate minimal solution, individualized according to the differing growth requirements of the

specific organism. The vials were swirled and rolled for thirty seconds to ensure contact with all lypohilized cells and 0.5 ml transferred to 4.5 ml appropriate dilution medium for titer. One ml serial dilutions were performed in 9 ml dilution medium and 0.1 ml aliquots were spread plated in duplicate. Plates were incubated at room temperature and checked at 48 and 72 hours to determine recovery.

The wash procedure and the wash and resuspension media used in the lyophilization experiments were designed to minimize carryover of non-essential materials into the lyophilized cells while maximizing cell recovery. Unlabeled lactate was used in each of these solutions to ensure that cellular processes necessary for lactate metabolism would be activated. Further, the presence of lactate would not interfere with the repeated addition experiments using ^{14}C-labeled lactate.

Results of lyophilization studies with the Ps-3 Isolate are presented in Table 16. In this study, four minimal media were selected and each was used both as wash and resuspension medium for any given lyophilized culture. Each of these four media contained lactate either at 0.1% or at 7.5% with and without added salts (0.1% $NaNO_3$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% KH_2PO_4; note that 0.1% lactate plus these salts constitutes Medium A). Results with these four media were

Table 16

Lyophilization and Resuspension of Ps-3 in Various Media

Freezing and Resuspension Media	Plating Media	Number of Cells Lyophilized	Number of Cells Recovered*	Log Loss per Vial	Average Log Loss
7.5% Lactate Solution	Lactate min(A)	1.23×10^7	2.5×10^2 1.3×10^3	4.69 3.98	4.34
7.5% Lactate and Salts‡	"	2.4×10^7	2.5×10^3 2.6×10^6	3.98 3.97	3.78
0.1% Lactate Solution	"	1.09×10^8	1.97×10^6 1.99×10^6	1.75 1.74	1.74
0.1% Lactate and Salts	"	8.6×10^7	5.4×10^6 3.0×10^4	1.20 3.45	2.32
7.5% Glucose	7.5% Glucose Agar	9.4×10^8	4.0×10^7 5.2×10^7	1.07 0.95	1.01

*Determinations were made on lyophilized cells after 20 hours storage at room temperature. Lyophilization vials were broken and cells resuspended and plated in the indicated media.

‡ Salts = 0.1% $NaNO_3$, 0.02% $MgSO_4$, and 0.02% KH_2PO_4.

compared to those obtained using a rich medium containing 7.5% glucose in nutrient broth. The results show low recovery rates for those cells lyophilized and resuspended in 7.5% lactate with or without added salts. Highest recovery rates were obtained with the rich glucose medium, as anticipated, since these cells showed a viability loss of only one order of magnitude. Cultures treated in 0.1% lactate showed losses of approximately two orders of magnitude, somewhat less than those observed with the rich glucose medium but considerably above those with 7.5% lactate. The influence of added salts on the recovery rates with 0.1% lactate was not established, however. These experiments demonstrate the feasibility of using a minimal medium for cell preservation. Although recovery in 0.1% lactate is less than that observed with the enriched medium, the procedure with 0.1% lactate nonetheless preserves a population sufficient for a BWS inoculum.

Similar experiments have been conducted with Pseudomonas fluorescens. Because of the possible identity of P. fluorescens with Isolate Ps-3 (see previous section), it was predicted that 0.1% lactate as both wash and resuspension media would produce similar recovery rates to those obtained with Ps-3. The results (Table 17) show losses of about two orders of magnitude, a result comparable with that obtained for Isolate Ps-3. Further, the presence of salts (those used

Table 17

Lyophilization and Resuspension of Pseudomonas fluorescens in Various Media

Freezing Media	Resuspension Media	Plating Media	Number of cells Lyophilized	Average Number of cells Recovered	Average Log Loss
0.1% Lactate	0.1% Lactate	Lactate min	4.0×10^7	2.40×10^5	2.26 ± 0.19
0.1% Lactate and salts‡	0.1% Lactate and salts	Lactate min	1.8×10^7	$1.57 \times 10^{5*}$	2.10 ± 0.14*
0.1% Lactate	0.1% Lactate and salts	Lactate min	4.0×10^7	6.8×10^6	0.76 ± 0.03
0.1% Lactate	0.1% Lactate and salts	Lactate min	1.16×10^5	1.03×10^4	0.78 ± 0.18

*Determinations were made on lyophilized cells after 20 hours storage at room temperature. Vials were broken, and the cells resuspended and plated in the indicated medium.

‡Salts = 0.1% $NaNO_3$, 0.02% $MgSO_4 \cdot 7H_2O$, and 0.02% KH_2PO_4

above) with 0.1% lactate in both the wash and resuspension medium had no significant effect on recovery.

In a further attempt to maximize recovery with a minimal medium, additional experiments were conducted with P. fluorescens in which the wash medium differed from the resuspension medium. The wash medium was retained at 0.1% lactate to minimize carryover of constituents of the growth medium. However, the resuspension medium contained both 0.1% lactate and added salts to maximize recovery and growth. The results (Table 17) show that this procedure considerably enhances recovery from the lyophilization process. The losses observed are less than an order of magnitude, lower than those observed with the enriched medium used with Isolate Ps-3. That this recovery rate is independent of initial cell concentration is indicated by the further finding that initial inoculums differing by more than two orders of magnitude produced similar recovery rates. These results indicate that since low cell concentrations successfully survive the procedure, it would be unnecessary to dilute a more concentrated inoculum prior to its use in a metabolic experiment. Rather, a series of lyophilized ampoules could be prepared, each of which contained the desired experimental titer.

Lyophilization studies with Azotobacter chroococcum also utilized the 0.1% lactate wash medium. However, two

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resuspension media were considered. One consisted of 0.1% lactate with salts (as above) plus a trace of molybdenum (Medium N):

Medium N: *Azotobacter* Lyophilization Medium with Nitrate

Sodium Lactate	0.1% (W/V)
Na_2MoO_4	trace
$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4

However, since this organism as been proposed in the BWS for nitrogen fixation experiments, resuspension in a similar medium lacking nitrate (Medium O) was also investigated:

Medium O: *Azotobacter* Lyophilization Medium without Nitrate

Sodium Lactate	0.1% (W/V)
Na_2MoO_4	trace
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4

The results (Table 18) indicate that the nitrate supplement to the resuspension medium has no significant effect on recovery. In both cases, losses of approximately four orders of magnitude were observed, considerably less than the recoveries observed for Ps-3 and *P. fluorescens*. That storage time, at least on a short term basis, does not impact this result is shown by essentially identical

Table 18

Lyophilization and Resuspension of Azotobacter in Various Media

Freezing Media	Resuspension Media	Plating Media	Number of Cells Lyophilized	Hours Stored	Number of cells Recovered	Average Log Loss
0.1% Lactate	0.1% Lactate and salts‡	Lactate min	3.3×10^7	20	8.8×10^2	4.28 ± 0.04
0.1% Lactate	0.1% Lactate and N-free salts *	N-free Lactate min	3.3×10^7	20	8.2×10^2	4.31 ± 0.07
0.1% Lactate	0.1% Lactate and N-free salts *	N-free Lactate min	3.3×10^7	164	8.0×10^2	4.32 ± 0.07**

‡Salts = 0.1% $NaNO_3$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% KH_2PO_4 and trace Na_2MoO_4

*N-free salts = 0.02% $MgSO_4 \cdot 7H_2$), 0.02% KH_2PO_4 and trace Na_2MoO_4

**Average of 2 vials; all others average of 3 vials.

recoveries from cultures examined either 20 or 164 hours following lyophilization. However, although the recoveries are low for this culture, the technique can nonetheless provide a population sufficient to serve as a BWS inoculum, particularly if the losses can be compensated by using higher initial inoculums. Alternately, since high recoveries can be obtained for other cultures, perhaps a different combination of wash and resuspension media could improve the recovery.

The culture used for the Azotobacter experiment was a 72 hour culture grown on Medium O presumably containing both cysts and vegetative cells. In an attempt to account for the low recovery, an additional experiment was conducted to determine whether cysts and vegetative cells differed in survival rates. Vegetative cells were obtained by loop inoculation of a nitrogen-free medium (Medium O) which was incubated for 24 hours whereas cysts were obtained from a similar culture incubated for 96 hours. Phase contrast microscopy was used to confirm the preponderance of cysts in the 96 hour culture. The results (Table 19) of a lyophilization experiment comparing survival of both cultures show that only the cyst form survives, but at a low rate.

Losses from application of the lyophilization technique to Rhodospirillum rubrum are as severe as those from Azotobacter

Table 19

Comparison of Stability of Azotobacter Vegetative Cells and Cysts to Lyophilization

Azotobacter culture form	Freezing Media	Resuspension Media	Plating Media	Age of Culture Lyophilized (Hours)	Number of Cells Lyophilized	Number of cells Recovered	Average Log Loss
Mixed (Vegetative and Cysts)	0.1% Lactate	0.1% Lactate and Salts†	Medium A Lacking NO_3	72	3.3×10^7	8.2×10^2	4.3 ± 0.07*
Vegetative	0.1% Lactate	0.1% Lactate and Salts†	Medium A Lacking NO_3	24	1.8×10^6	0	6
Cysts	0.1% Lactate	0.1% Lactate and Salts†	Medium A Lacking NO_3	96	1.3×10^7	5.1×10^3	4

†Salts = 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% KH_2PO_4 and trace Na_2MoO_4

*Based on 2 reaction vials; all other averages based on triplicates.

(Table 20) using the 0.1% lactate wash medium and a resuspension medium containing 0.1% lactate, salts (as above), and trace amounts of biotin (Medium J). Attempts to improve recovery were made utilizing a defined enriched medium (Medium I) for resuspension. However, comparable losses of four orders of magnitude were also observed with this medium. Nonetheless, as with Azotobacter, sufficient cells survive to serve as a BWS inoculum.

In summary of these studies, then, the results demonstrate the applicability of lyophilization to the proposed BWS. It should be noted, however, that while the technique is successful with bacteria, Corbett and Parker (19) report survival of blue-green algae only when lyophilized with serum. Attempts to substitute a more simplified medium were unsuccessful. Future studies for the BWS must therefore pursue a means of preserving and re-vitalizing algae in a manner compatible with the BWS.

5.2 Preservation in an Active State

An alternative method for culture preservation is to maintain the culture in an active state with growth and metabolism occurring throughout the duration of its use in the Basic Warning System. Standard laboratory methods utilize periodic culture transfers or continuous cultivation. However, the manipulations required for automating transfers and monitoring serial cultures are pro-

Table 20

Lyophilization and Resuspension of Rhodospirillum rubrum in Various Media

Freezing Media	Resuspension Media	Plating Media	Number of Cells Lyophilized	Number of Cells Recovered	Average* Log Loss
0.1% Lactate	0.1% Lactate and Salts‡	Medium J	4×10^8	4.4×10^3	4.66 ± 0.05
0.1% Lactate	Complete (Medium I)	Complete (Medium I)	4×10^8	1.8×10^4	3.93 ± 0.05

‡Salts = 0.1% $NaNO_3$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.02% KH_2PO_4 and trace Biotin (Medium J)

*Based on Duplicate Vials.

hibitive as is the volume of culture media required. Similar disadvantages accompany continuous cultivation and all such techniques suffer from possible genetic variation usually accompanying such long term maintenance.

An interesting variation for maintaining an active culture is use of the repeated addition technique whereby a limiting amount of substrate is added to a viable culture at specified time intervals. Metabolism of the added substrate should result in cell growth. As the substrate is exhausted, cell growth ceases and the culture enters stationary phase. In order to maintain the culture viable, additional substrate must then be made before or shortly after the onset of death phase. Assuming that the response to each substrate would be uniform and monitored throughout the duration of the operation of the BWS, this approach has the advantage of long-term establishment of a culture's response to substrate in the absence of added inhibitors. This would provide a highly reliable statistical basis for analyzing effects of a Mars sample on vital terrestrial processes.

The repeated addition technique has been studied extensively with Isolate Ps-3 using ^{14}C-labeled lactate as substrate (see Section 3.1.1). As discussed previously, these experiments showed that a second substrate addition results in a metabolic response comparable to the first

response (Figure 2). Similar responses can also be obtained from further repeated substrate additions. Other experiments reported earlier indicated that cultures in such experiments gave reduced responses after 265 hours. This implies that long term culture maintenance would require substrate additions at intervals somewhat less than 265 hours.

In order to determine the applicability of the repeated addition technique to long term maintenance of an active culture, an experiment has been conducted with Isolate Ps-3 and ^{14}C-labeled lactate in which substrate additions were performed at one week (app. 168 hours) intervals over a four month period. Substrate concentrations were maintained low to ensure that each response increment is within the dynamic range of the measurements and to prevent build-up of any toxic metabolic by-products. Substrate utilization was monitored by $^{14}CO_2$ evolution using experimental procedures as described for earlier experiments.

The results (Table 21) show that a uniform cultural metabolic response can indeed be maintained by this technique throughout the duration of the experiment. An additional experiment in which cell numbers were determined prior to and following each weekly repeated addition over a six week interval showed a similar metabolic result (Figure 19). Concurrent with the metabolic response, cell numbers were

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Table 21

Response to Repeated Additions of ^{14}C-labeled Lactate to Ps-3

Addition	Measured Amount Added Adjusted to First Addition as Base*	$^{14}CO_2$ Evolved (cpm)	Calculated $^{14}CO_2$ Evolved (cpm)	Relative Response* (%)
1	1.00	6156	6156	100
2	.69	4836	7009	114
3	.73	3190	4370	71
4	.68	3830	5632	91
5	.94	5647	6007	98
6	.93	6579	7074	115
7	.96	4484	4671	76
8	.91	4223	4641	75
9	.87	5085	5845	95
10	.96	4525	4714	76
11	.88	5792	6582	107
12	.85	6073	7145	116
13	.96	6447	6718	109
14	.92	6700	7283	118
15	.97	6812	7023	114
16	.96	7154	7452	121
17	.98	7334	7484	121
18	.98	6271	6399	104
10	1.00	6079	6079	99
20	.91	4826	5303	86
21	.96	4338	4519	73

Each addition of 0.04 μCi Lactate was added in a 0.05 ml volume to an initial volume of 0.05 ml (10^3 cells) on a silica base.

*Each subsequent value has been recalculated using the initial addition as the base.

Sample Calculation

$$\frac{^{14}CO_2 \text{ Evolved in Subsequent Addition}}{\text{Amount of Label Added/Initial Amount of Label Added}} = \frac{4836}{.69} = 7009$$

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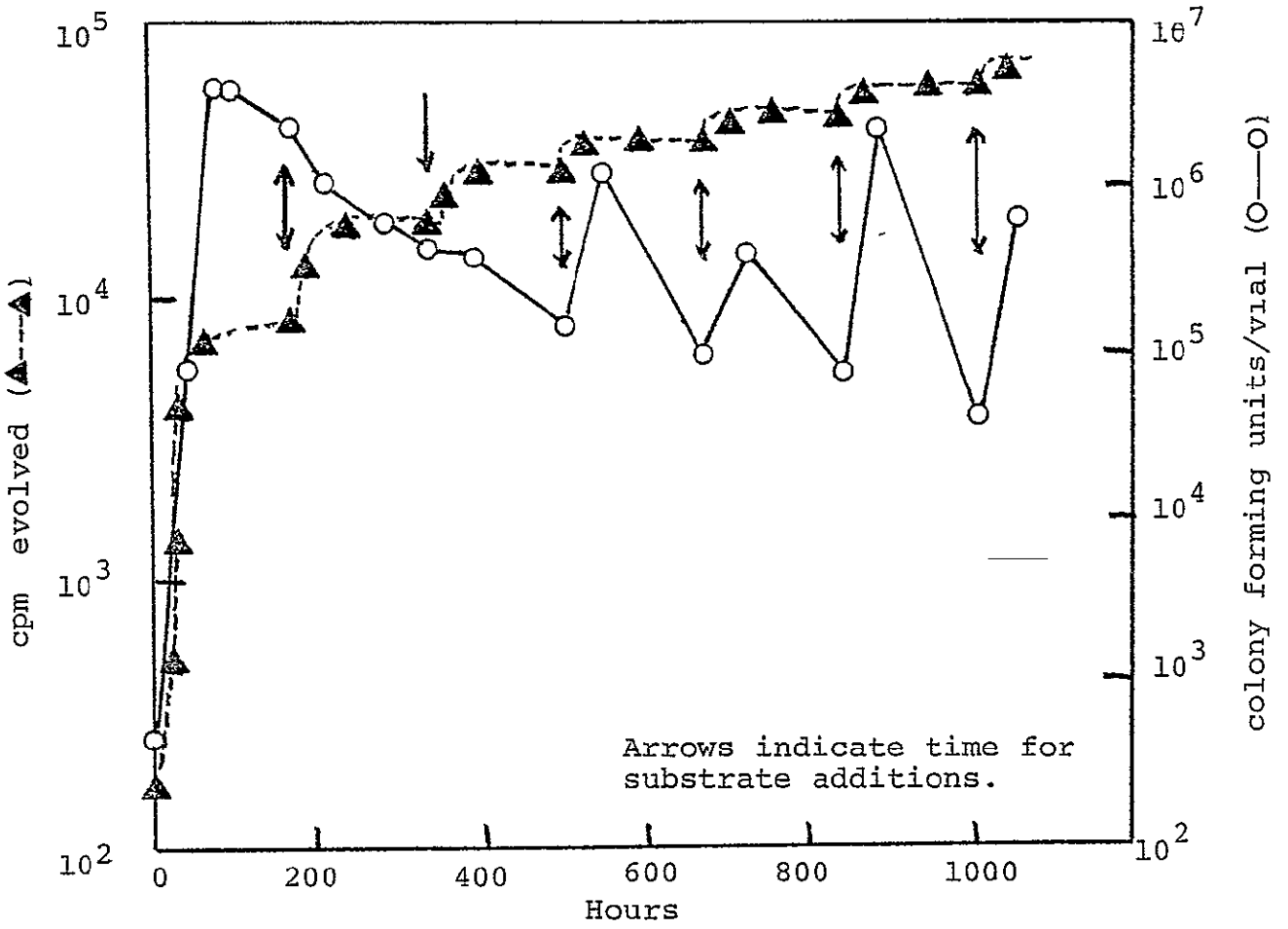
Figure 19

Effect of Repeated Additions of ^{14}C-Lactate on Ps-3 Metabolism and Cell Enumeration



observed to increase and then decrease as substrate was consumed (Figure 19). The net result of this cyclic change in population size was overall maintenance of a relatively stable population size. These results support the repeated addition concept for long term maintenance of an active culture. Provided the system would continue for three or more years, the repeated addition technique could thus serve as a means for culture preservation.

Despite the statistical advantages of maintaining a viable culture, the repeated addition approach to cell preservation has several disadvantages. Large quantities of labeled substrate are required to perform many additions over three or more years. This necessitates considerable instrument usage for performing and monitoring the additions, which in turn requires high mechanical reliability. Another limitation arises from the potential spontaneous genetic changes accompanying DNA replication that could change the established culture characteristics.

Another approach to culture maintenance in an active state is through the use of a balanced ecosystem. System maintenance would be dependent upon cycling ^{14}C-labeled carbon between heterotrophs and autotrophs. The integrity of the ecosystem would be monitored by $^{14}CO_2$ levels in the headspace above the reactants. This concept eliminates some

of the disadvantages of the repeated addition technique by eliminating the need for substrate storage and addition. However, it preserves the statistical advantage derived from long-term monitoring to establish an ecosystem base-line metabolic pattern.

Preliminary ecosystem experiments performed using the Ps-3 Isolate and Nostoc demonstrated an initial heterotrophic release of $^{14}CO_2$ followed by a rapid autotrophic uptake of $^{14}CO_2$ (Figure 10). To test the applicability of the ecosystem for long term cell preservation, ecosystem cultures were maintained for one month and monitored for culture viability. At the end of this period, algal cultures showed loss of green color and cytoplasmic deterioration. Bacterial cultures showed loss of green color and cytoplasmic deterioration. Bacterial cultures showed increases in cell number over the first 72 hours, then decreases corresponding to a death phase. Thus, the experiments were unable to extend culture life sufficiently to consider the current ecosystem concept as a technique for long term culture preservation. Further efforts for long term maintenance of an active culture will utilize the repeated addition technique.

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6.0 CONCEPT FOR BASIC WARNING SYSTEM BREADBOARD

The key features defining an instrument for the BWS are shown in Table 22. The next step in the development towards such a system would be the fabrication of a functional breadboard.

It is suggested that Labeled Release-type hardware used in the Viking System be adapted to the BWS to the extent feasible. A breadboard could readily be obtained by modification of the existing Labeled Release (LR) Test Standards Module (TSM) to provide a functional BWS. A review of the features of Figure 20 will indicate the changes required for the TSM. The TSM could accomodate and dispense the substrate(s) without any modifications. Similarly, the helium gas purging system is satisfactory. The minor adaptation to supply a "Mars" atmosphere to the test cell would require only a change of the gas tank from CO_2 to a Mars atmosphere tank. Mars atmosphere is then supplied by pumping down the glass enclosed environmental chamber to a total pressure of less than 0.1 millibar. At this point, a valve to the "Mars" atmosphere tank could be opened to fill the test chamber. The valve from the "Mars" atmosphere tank could then be closed and the test chamber pumped down to its operational range of 10-100 millibars, depending on the particular experiment. During

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Table 22

Basic Warning System Key Features

A. Test Components Requiring Storage

	Component	Storage Conditions
1.	^{14}C substrate(s)	Sterile aqueous solution in sealed ampoule(s)
2.	Purge gas for substrate(s)	Sterile in pressurized tank
3.	"Mars" atmosphere	Sterile in pressurized tank
4.	Water	Sterile in sealed ampoule(s)
5.	Earth organisms in soil	Lyophilized in sealed ampoule(s)
6.	Mars soil sample	Aseptic acquisition, quarantine

B. Test Modes

1. Controls

   a. Substrate alone
   b. Substrate with Earth organisms
   c. Substrate with pre-humidified Mars sample
   d. Substrate with pre-sterilized Mars sample
   e. Substrate with Mars sample

2. Interactive

   a. Substrate with Mars sample plus Earth organisms -- repeated additions
   b. Substrate with pre-humidified Mars sample plus Earth organisms -- repeated additions

C. Special Requirements

1. Sterilizability of all system components
2. Temperature control (-50°C to +5°C)
3. Atmospheric composition (95% CO_2, 0.2% O_2, 2% N_2)
4. Atmospheric pressure (10-100 mb)
5. Humidity (TBD)
6. Three year storage capability for all Earth components
7. One year maintenance and storage capability for Mars sample
8. Solar simulator lamp (Mars version)

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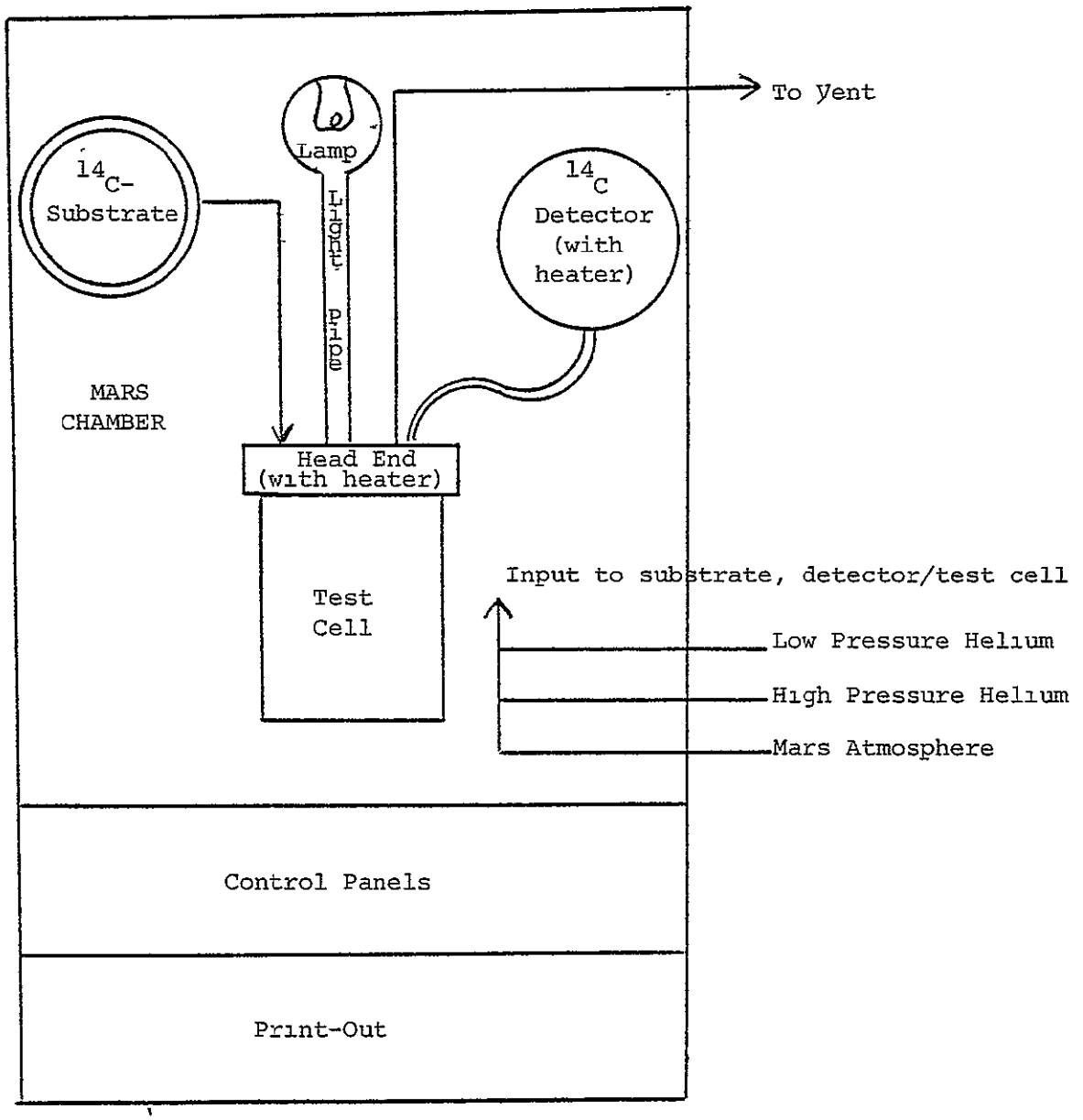
Figure 20

Schematic of BWS Breadboard Based On
Modification of LR TSM



Required changes involve incorporation of a Lamp and Light Pipe through the test cell head end assembly and substituting a supply of Martian Atmosphere to the LR test cell in place of the CO_2 supply.

these operations, the test cell would have been kept open so that its atmosphere would be the same as that in the test chamber. This procedure is completely analogous to the procedure currently used to obtain CO_2 at 5 torr in the LR test cell.

In order to provide pre-humidification of the Mars sample in the test cell, a water reservoir should be added to the system. A humidity sensor will also be required. This addition requires little modification since the TSM originally was fitted with a humidification chamber that was later omitted. This water reservoir should be capable of supplying either liquid water to the test chamber or water vapor only through evaporation at controlled rate. Liquid water could be added through the "metered" cavity for the nutrient injection system whereas water vapor could be added simply by equilibrating the headspace of the test cell with that of the water chamber. In this mode, the vapor pressure in the test cell will be that of water at the test cell temperature. Using a humidity sensor, the correct amount of water vapor in the environmental chamber of the TSM might be approached by injecting nutrient into an open dump cell and permitting the water to evaporate into the test chamber atmosphere. The desired relative humidity might then be achieved by pump down of the atmosphere from the "Mars" atmosphere tank. In either case,

pre-humidification of the Mars sample might be accomplished by exposing the Mars soil in a test cell open to the test chamber atmosphere to permit equilibration with the water vapor. When the proper equilibrium point had been reached, the sample could be exposed for the desired length of time.

For experimental purposes in the BWS breadboard, the terrestrial test species of microorganisms prepared in natural or artificial soil, could be stored in lyophilized form within sealed glass ampoules. These glass ampoules could be broken immediately prior to use at a point adjacent to the BWS breadboard in order to minimize exposure to room atmosphere and humidity. It is believed this would prove adequate for test purposes. However, if it were necessary to eliminate exposure to the room environment completely, a modification of the TSM test cell would be required to adapt it to receive a sealed glass ampoule containing the soil-microorganism mixture. A mechanism would have to be provided whereby, when the environmental chamber is in operating status, a remotely controlled mechanism could seal the test cell and break the ampoule within the sealed test cell.

In BWS breadboard tests, Mars organisms would be simulated by various terrestrial organisms selected for predation or other interactions with the BWS organism.

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The above described system would permit operation of the vital processes test system in the heterotrophic mode only. To permit phototrophic mode operation, a lamp would have to be added to the system. Perhaps the easiest way to achieve this would be through the mounting of a xenon lamp with the spectral characteristics used in the Pyrolytic Release Viking life detection experiment, within the Mars chamber, close to the test cell. A light pipe might then be used to deliver a beam of light onto the soil in the test cell by cutting a window in the test cell and sealing the light pipe to it. Duty cycle of the lamp could be controlled externally.

The above BWS breadboard could also be adapted to permit testing of hardy organisms under environmental stress. At the moment, the hardy organism system is operating at 15^{o}C, a temperature readily attainable by the current TSM. However, if at a later point temperatures less than 10^{o}C are desired, this could be accomplished by the addition of a temperature control system, probably a thermo-electric cooler attached to the test cell. The low atmospheric pressure under which the experiments would be conducted would help to insulate the test cell from thermal radiation from the room. If needed, a reflective shield could be placed around the test chamber. Thermal

insulators might be introduced to interrupt thermal conductive pathways to the instrument.

It is believed that the relatively simple modification of the existing TSM in the manner described above would provide a BWS breadboard capable of functioning in all the test modes listed in Table 22. The dynamic range of the detector system would permit multiple repeated addition experiments to be performed. However, should the total amount of radioactivity evolved in a single experimental series exceed the capacity of the counting system, the specific activity of the substrate(s) could be reduced. Alternatively, the test cell could be vented to remove radioactive gas from the system.

While the above breadboard will permit terrestrial evaluation of the BWS, significant design changes will be necessary for flight hardware. Whereas the TSM and the Viking hardware operate under gravity, the BWS will operate in an essentially weightless environment. This will require development of new techniques for the handling of soil, liquid, and gas within the instrument.

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7.0 CONCLUSIONS AND RECOMMENDATIONS

This year's effort was devoted almost exclusively to further development of a Basic Warning System using terrestrial indicator organisms. In preliminary fashion, this study has indicated the feasibility of providing a system capable of early monitoring of possible hazardous effects on Martian organisms on terrestrial biology. It has been demonstrated that the system can monitor interactions over an extended period of time through the use of repeated additions of substrates. The system may be used for both heterotrophic and phototrophic process monitoring.

The Basic Warning System has also been developed from the viewpoint of detecting effects of extraterrestrial organisms in the presence of a variety of possible artifacts. Thus, the LR data from the Viking '76 Mission to Mars showed an initial rapid evolution of radioactive gas upon injection of substrate to the sample. After seven sols, however, upon addition of a second nutrient injection, there was an actual dimunition of the total radioactivity present in the headspace of the test cell (3). The absence of additional evolution of radioactivity upon second substrate injection to the Mars sample could reflect inactivation of the reactant responsible for the initial reaction, possibly by water vapor. It should be noted that the proposed BWS has been designed to

yield gas evolution with each substrate addition despite the dimunitions seen with the Mars sample. Thus, in a previous report (1), it was demonstrated that the magnitude of each response with ^{14}C lactate and terrestrial organisms was sufficiently large to be detected even after subtraction of the dimunition that was obtained on Mars with a Martian sample. Thus, should a reactant pose a threat to terrestrial biological processes, this should become apparent during the monitoring of the extended additions provided by the BWS. The controls also permit a determination of whether any reactant in the Mars sample is humidity labile.

To date, the BWS system has been developed to the point of initiating extreme environmental experiments in the Mars box. Additional experiments of this type should be conducted in the future with a view toward defining the system more precisely. Specific protocols for long duration, repeated addition experiments in the hardy organism mode should be developed to the level of that developed for the vital processes mode.

By the time this laboratory effort is accomplished, Viking Mission requirements for use of the TSM will probably have been completed. If so, the TSM should be adapted as described in this report to serve as a breadboard BWS. Experiments with the breadboard should be carried out with an eye toward developing flight hardware design.

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A review should be made of the quarantine protocol, the sample acquisition, and the sample handling engineering concepts developed over the past few years within this program. The concepts should be updated, particularly in view of the Viking mission data, and, where appropriate, should be further elaborated with an eye towards being incorporated into a return Mars sample mission.

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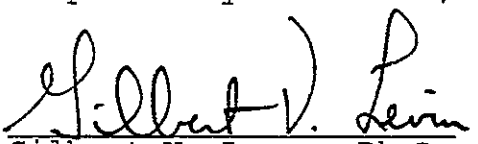
Respectfully submitted,



Gilbert V. Levin, Ph.D.
Principal Investigator

8.0 ACKNOWLEDGMENTS

Dr. Jayne Carney was responsible for the detailed design and execution of the microbiological experiments undertaken in testing the concepts of the Basic Warning System. Upon the departure of Dr. Carney from Biospherics, Dr. Patricia Ann Straat reviewed, analyzed, and summarized the microbiological data reported herein. The excellent technical assistance of Kim Schroader in performing most of the cited experiments is gratefully acknowledged.

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9.0 REFERENCES

1. "Technology for Planetary Return," Annual Report for Contract No. NASW-2856, Prepared for NASA Headquarters, Biospherics Incorporated, Washington, D.C. (1976).

2. Klein, H.P., N.H. Horowitz, G.V. Levin, V.I. Oyama, J. Lederberg, A. Rich, J.S. Hubbard, G.L. Hobby, P.A. Straat, B.J. Berdahl, G.C. Carle, F.S. Brown, and R.D. Johnson, Science, 194, 99 (1976).

3. Levin, G.V. and P.A. Straat, Science, 194, 1322 (1976).

4. Levin, G.V. and P.A. Straat, J. Geophys, Res., 87, 4663 (1977).

5. Levin, G.V. and P.A. Straat, Origins of Life, 7, 293 (1976).

6. Levin, G.V., Icarus, 16, 153 (1972).

7. Buddemeyer, E., Appl. Microbiol., 28, 177 (1974).

8. Novitsky, J., and R. Morita, Appl. and Environ. Microbiol., 32, 617 (1976).

9. Stewart, W.D.P., C.P. Fitzgerald, and R.H. Burris, Proc. Nat. Acad. Sci., U.S.A., 66, 1104 (1970).

10. Dagley, S. and P.J. Chapman in Methods in Microbiology, J.R. Norris and D.W. Robbins, Eds., Academic Press, New York, 1971, Vol. 6A, p. 228.

11. Dixon, M. and E.C. Webb, Enzymes, Academic Press, Inc. New York, 1958.

12. Stewart, W.D.P. in Annual Review of Microbiology, M.P. Starr, et al, Eds. Annual Reviews, Inc., Palo Alto, Calif., 1973, Vol. 27, p. 283.

13. Dawson, R.M.C., D.C. Elliott, W.H. Elliott, and K.M. Jones, Data for Biochemical Research, Oxford University Press, London, 1959.

14. Fruton, J.S. and S. Simmonds, General Biochemistry, Second Edition, John Wiley & Sons, Inc., New York, 1958.

15. Braun, G. and B.Z. Braun in Algal Physiology and Biochemistry, W.D.P. Steward, Ed. University of California Press, Berkley, 1974, p. 346.

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16. Oyama, V.I., B.J. Berdahl, and G.C. Carle, Nature, 265, 110 (1977).

17. Crowe, J.H. and A.F. Cooper, Jr., Scientific American, 225(6), 30 (1971).

18. Norris, J.R. and D.W. Robbins, Eds., Methods in Microbiology, Vol. 3A, Academic Press, New York, 1970.

19. Corbett, L.L. and D.L. Parker, Appl. and Environ. Microbiol., 32, 777 (1976).

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Appendix I: Media Summary

Medium A: Lactate Minimal Medium

Sodium Lactate	0.1% (W/V)
$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4

Medium B: Pseudomonas and Ps-3 Dilution Medium

$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4

Medium C: Standard Methods Agar

Bacto-Yeast Extract	2.5 g
Bacto-Tryptone	5.0 g
Bacto-Dextrose (Glucose)	1.0 g
Bacto-Agar	15.0 g

Medium D: Nitrogen Fixing Medium

K_2HPO_4	0.4 g
Na_2MoO_4	trace
$FeCl_3$	trace
Lactate	1.0%
Purified Agar	1.5%
Distilled Water	to 1 liter
pH	7.2

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Medium E: Nitrogen Fixing Dilution Medium

K_2HPO_4	0.4 g
Na_2MoO_4	trace
$FeCl_3$	trace
Purified Agar	1.5%
Distilled Water	to 1 liter
pH	7.2

Medium F: Nostoc Carbohydrate Medium #10

$NaNO_3$	.083 g
K_2HPO_4	0.01 g
$MgSO_4 \cdot 7H_2O$	0.025 g
$Na_2SiO_3 \cdot 9H_2O$	.027 g
$CaCO_3$	.040 g
Ferric Citrate	0.0035 g
Citric Acid	0.0035 g
Distilled Water	1.0 L
pH	8.0

Medium G: Combination Medium for Elementary Ecosystems

K_2PO_4	0.04 g
$MgSO_4 \cdot 7H_2O$	0.025 g
$Na_2SiO_3 \cdot 9H_2O$	0.027 g
$FeCl_3$	0.001 g
$CaCO_3$	0.02 g
Distilled Water	to 1 liter
pH	7.4

Medium H: Rhodospirillum Complete Medium (Van Niel's Yeast Extract Medium)

K_2HPO_4	1.0 g
$MgSO_4$	0.5 g
Yeast Extract	5.0 g
Tap Water	1.0 L
pH	7.0 to 7.2

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Medium I: Rhodospirillum Complete Defined Medium

KH_2PO_4	0.06%
K_2HPO_4	0.09%
$(NH_4)_2SO_4$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
$CaCl_2 \cdot 7H_2O$	0.008%
$FeSO_4 \cdot 7H_2O$	0.001%
DL-Malic acid	0.06%
EDTA	0.002%
H_3BO_3	280 µg/100 ml
$MnSO_4 \cdot 4H_2O$	210 µg/100 ml
$Na_2MoO_4 \cdot 2H_2O$	75 µg/100 ml
$ZnSO_4 \cdot 7H_2O$	24 µg/100 ml
$Cu(NO)_2 \cdot 3H_2O$	4 µg/100 ml
Biotin	1.5 µg/100 ml
pH	6.8

Medium J: Rhodospirillum Minimal Growth Medium

Sodium Lactate	0.1% (W/V)
$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Biotin	trace
Distilled Water	to volume
pH	7.2 to 7.4

Medium K: Rhodospirillum Dilution Medium

$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Biotin	trace
Distilled Water	to volume
pH	7.2 to 7.4

Medium L: Nutrient Broth

Peptone	5.0 g
Beef Extract	3.0 g
Distilled Water	1.0 L
pH	7.0

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Medium M: Azotobacter Dilution Medium

Na_2MoO_4	trace
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4

Medium N: Azotobacter Lyophilization Medium With Nitrate

Sodium Lactate	0.1% (W/V)
Na_2MoO_4	trace
$NaNO_3$	0.1%
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4

Medium O: Azotobacter Lyophilization Medium Without Nitrate

Sodium Lactate	0.1% (W/V)
Na_2MoO_4	trace
$MgSO_4 \cdot 7H_2O$	0.02%
KH_2PO_4	0.02%
Distilled Water	to volume
pH	7.2 to 7.4